

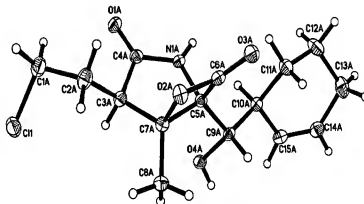
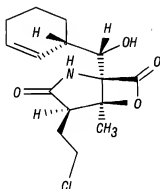
APPENDIX B



US 20050049294A1

(19) **United States**(12) **Patent Application Publication** (10) **Pub. No.: US 2005/0049294 A1****Palladino et al.**(43) **Pub. Date: Mar. 3, 2005**(54) **METHODS OF USING [3.2.0]
HETEROCYCLIC COMPOUNDS AND
ANALOGS THEREOF**(76) **Inventors:** Michael Palladino, Encinitas, CA (US);
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IRVINE, CA 92614 (US)(21) **Appl. No.: 10/871,368**(22) **Filed: Jun. 18, 2004****Related U.S. Application Data**(60) Provisional application No. 60/480,270, filed on Jun.
20, 2003. Provisional application No. 60/566,952,
filed on Apr. 30, 2004.**Publication Classification**(51) **Int. Cl.⁷** **A61K 31/407**
(52) **U.S. Cl.** **514/412**(57) **ABSTRACT**

Disclosed are methods of treating cancer, inflammatory conditions, and/or infectious disease in an animal comprising: administering to the animal, a therapeutically effective amount of a heterocyclic compound. The animal is a mammal, preferably a human or a rodent.



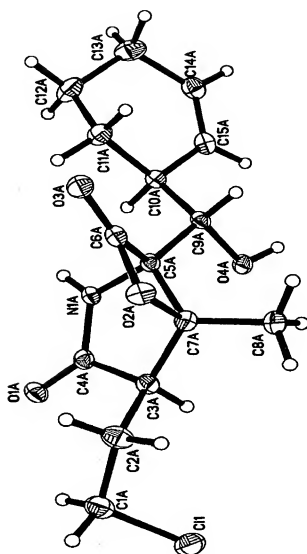
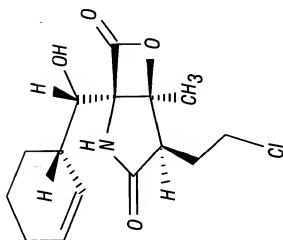


FIG. 1



Pan-tropical Distribution of the *Salinospora*



FIG. 2

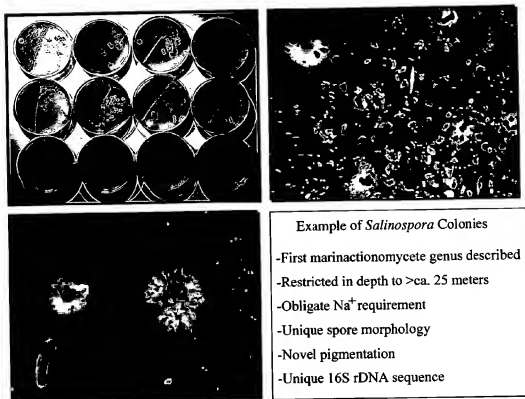
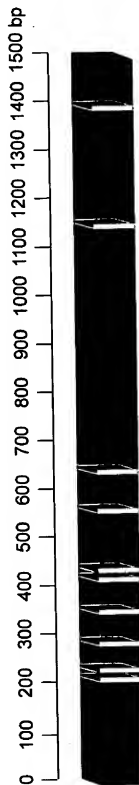


FIG. 3

Typical 16S rDNA Sequence of the *Salinospora*



Bars represent characteristic signature nucleotides of the *Salinospora* that separate them from their nearest relatives (*Micromonospora* species). >2% sequence divergence from nearest neighbor (*M. olivasterospora*)

FIG. 4

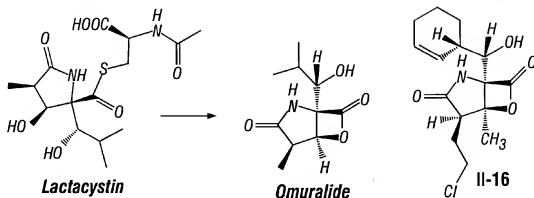


FIG. 5

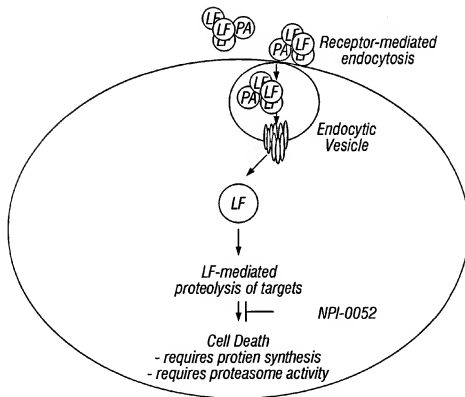
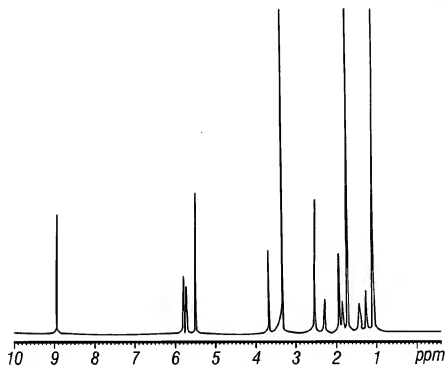
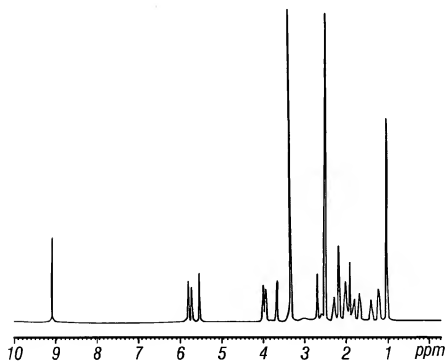


FIG. 6



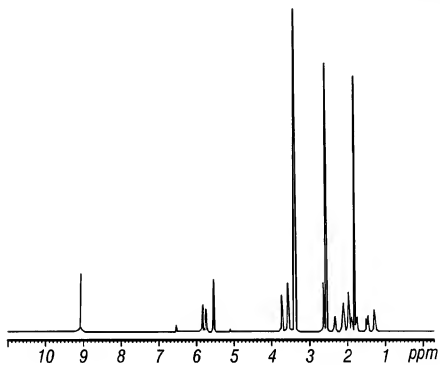
II-20

FIG. 7



II-24C

FIG. 8



II-19

FIG. 9

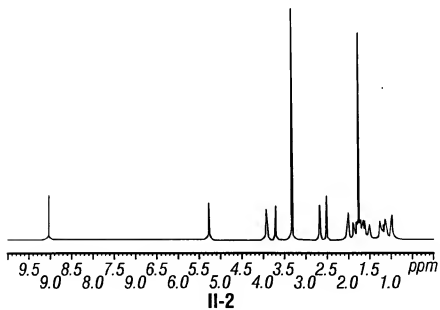
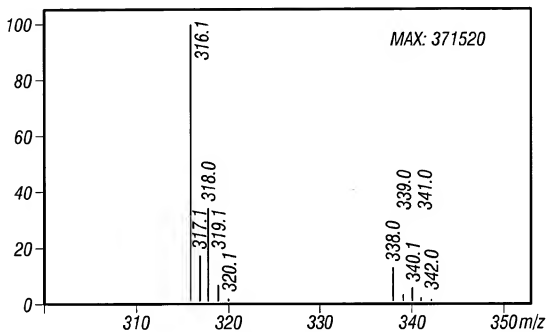
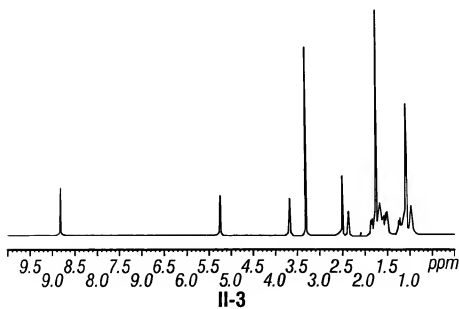
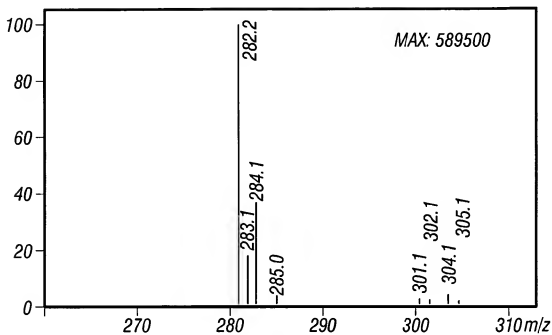
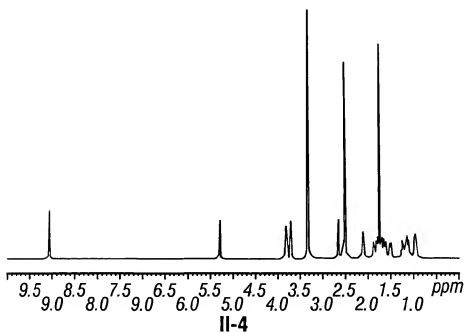
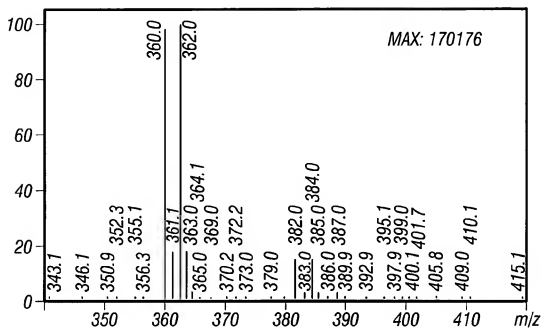
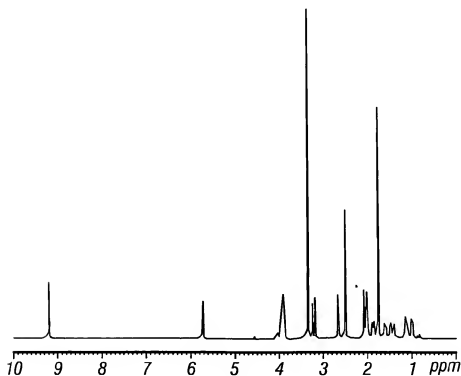


FIG. 10

**FIG. 11****FIG. 12**

**FIG. 13****FIG. 14**

**FIG. 15****II-5A****FIG. 16**

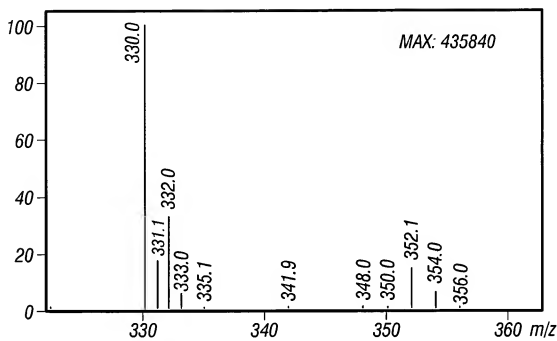
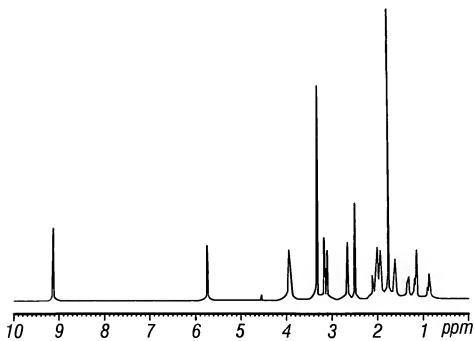


FIG. 17



II-5B

FIG. 18

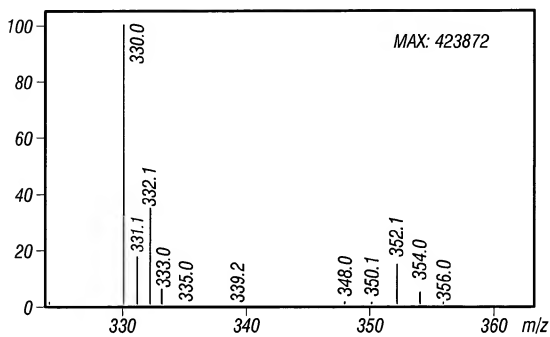
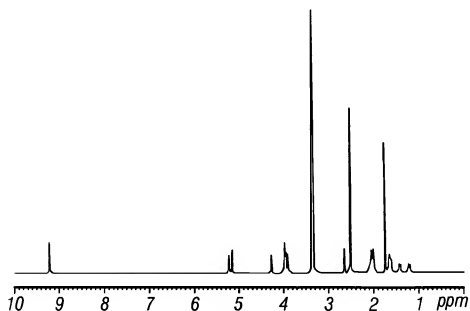
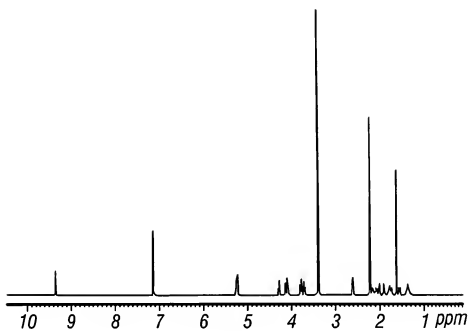


FIG. 19



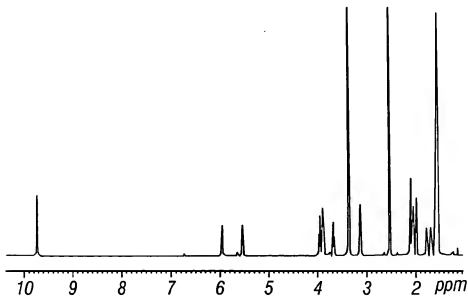
IV-3C

FIG. 20



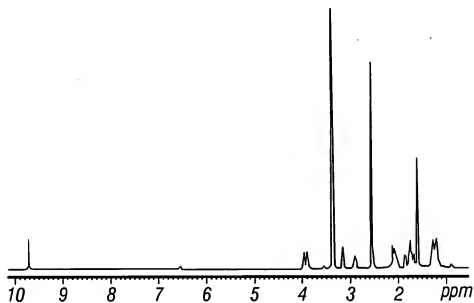
IV-3C

FIG. 21



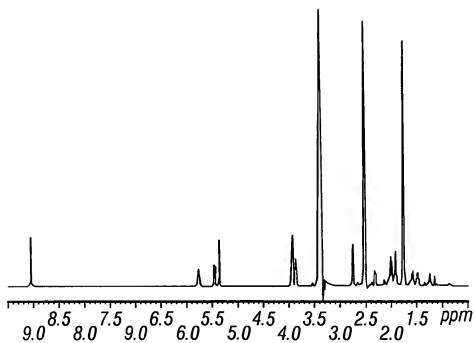
II-13C

FIG. 22



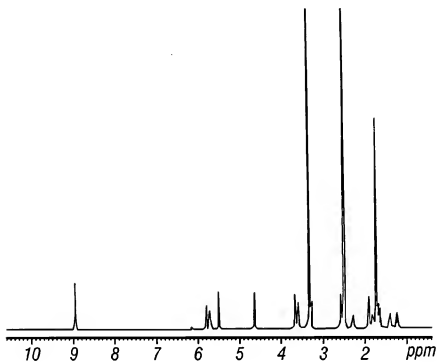
II-8C

FIG. 23



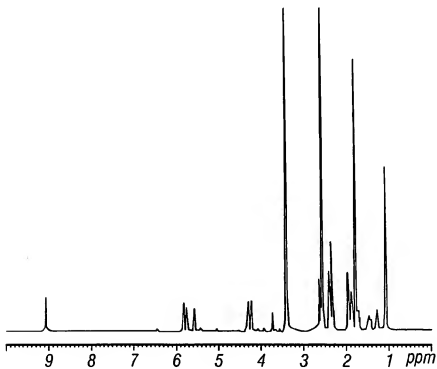
II-25

FIG. 24



II-21

FIG. 25



II-22

FIG. 26

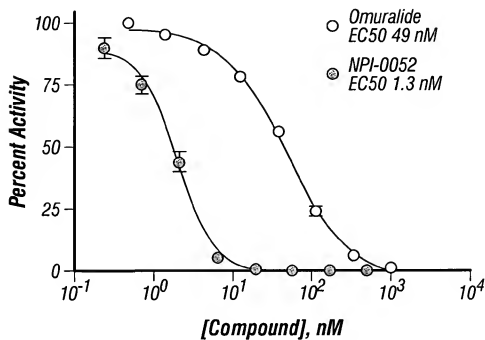


FIG. 27

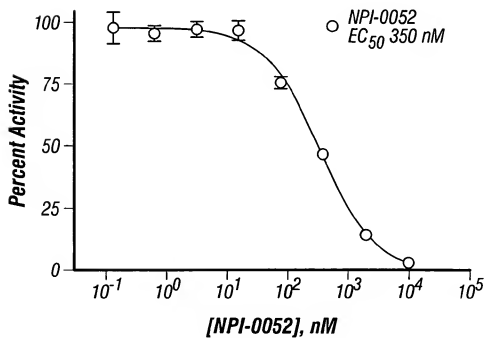


FIG. 28

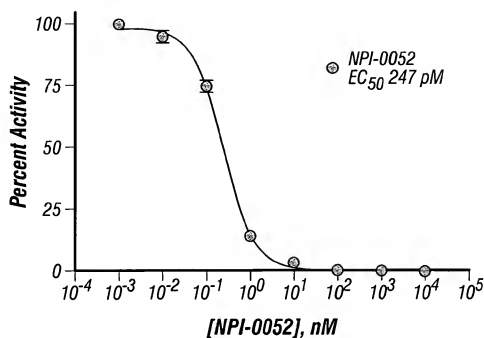


FIG. 29

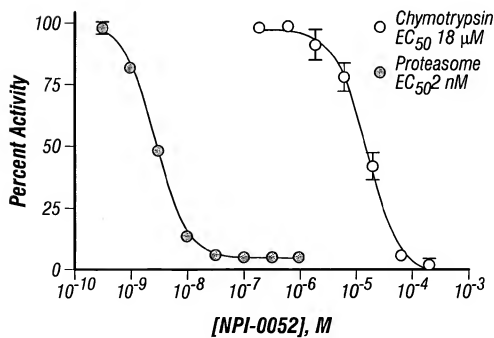


FIG. 30

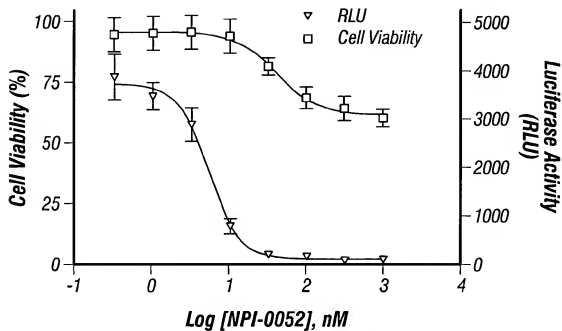


FIG. 31

Reduction of I κ B α degradation and Retention of Phosphorylated I κ B α by II-16 in HEK293 Cells (A) and the HEK293 NF- κ B/Luciferase Reporter Clone (B).

FIG. 32A

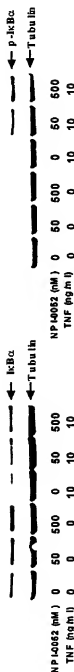
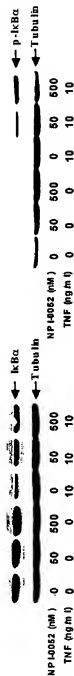


FIG. 32B



Accumulation of Cell Cycle Regulatory Proteins, p21 and p27 by IL-16 Treatment of HEK293 Cells (A) and the HEK293 NF- κ B/Luciferase Reporter Clone (B).

FIG. 33A

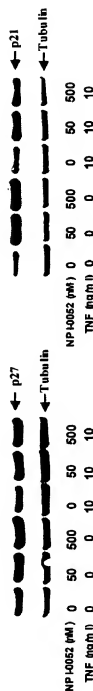
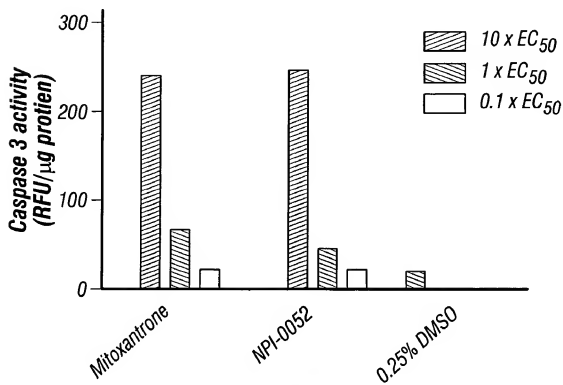
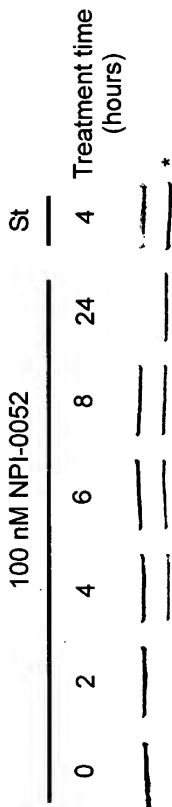


FIG. 33B



**FIG. 34**

PARP Cleavage by IL-16 in Jurkat Cells



¹ St; Staurosporine

FIG. 35

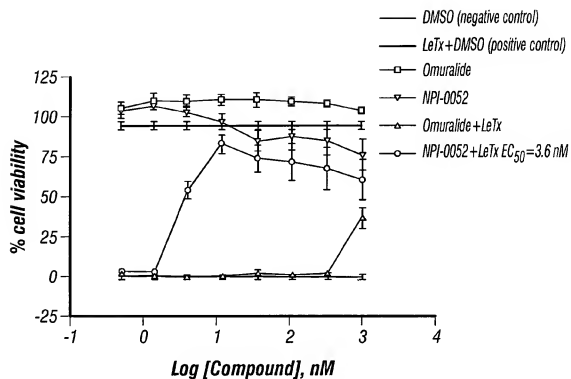


FIG. 36

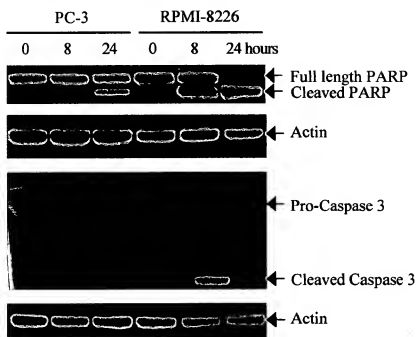


FIG. 37

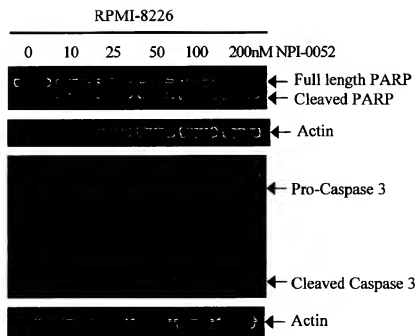
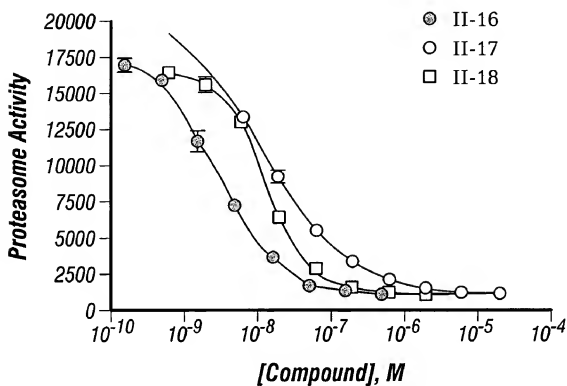


FIG. 38

**FIG. 39**

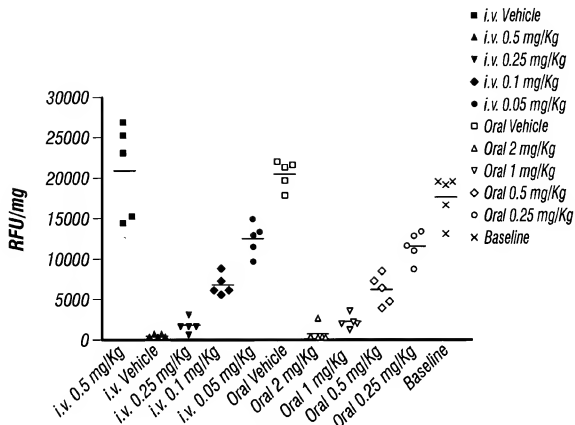


FIG. 40

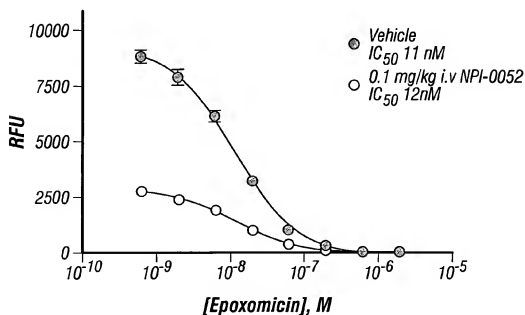


FIG. 41

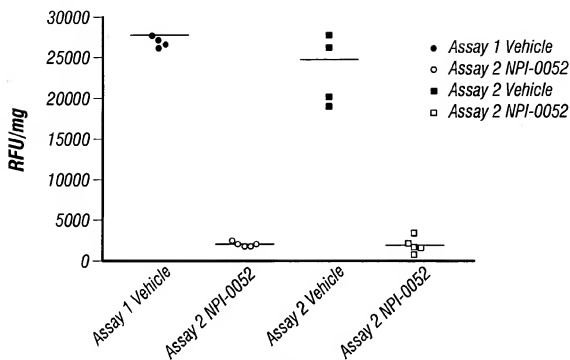


FIG. 42

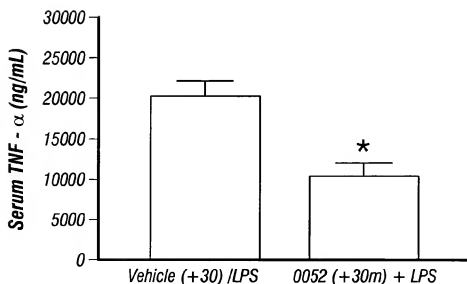


FIG. 43

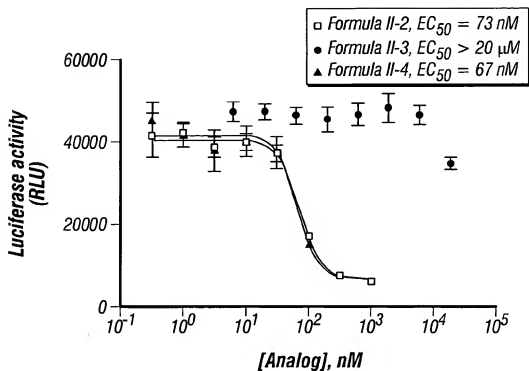


FIG. 44

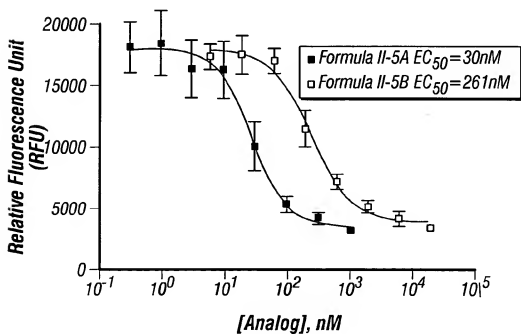


FIG. 45

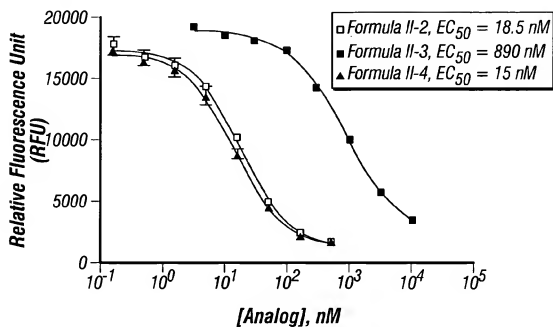


FIG. 46

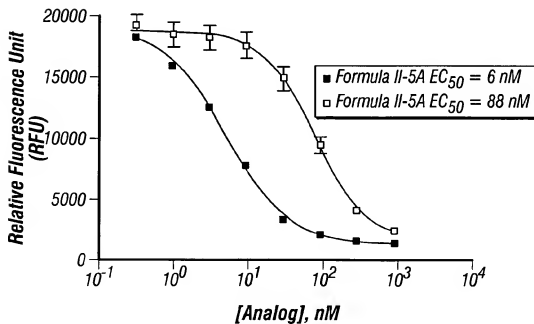


FIG. 47

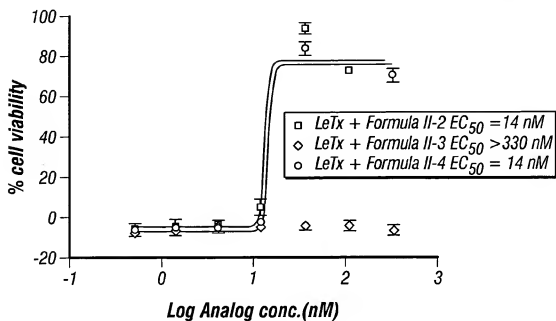
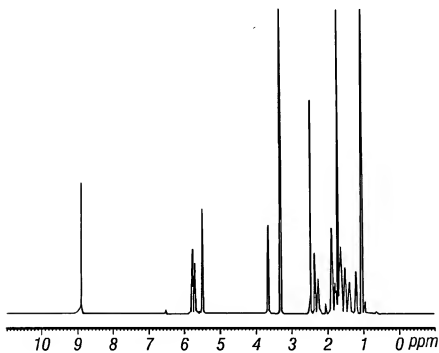
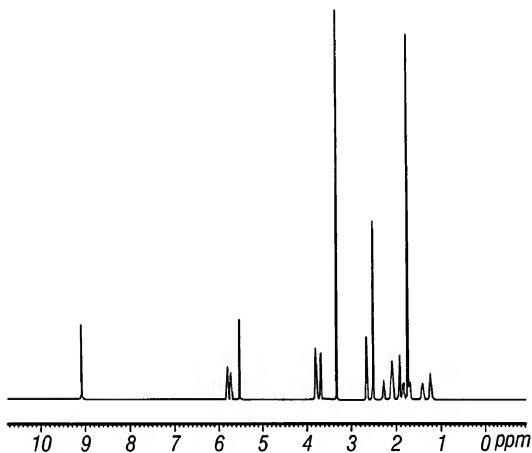


FIG. 48



II-17

FIG. 49



II-18

FIG. 50

METHODS OF USING [3,2,0] HETEROCYCLIC COMPOUNDS AND ANALOGS THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 119(c) to U.S. Provisional Application No. 60/480,270, filed on Jun. 20, 2003, entitled USE OF SALINOSPORAMIDE A TO TREAT LeTx INTOXICATION AND *B. ANTHRACIS* INFECTION, and to U.S. Provisional Application No. 60/566,952, filed on Apr. 30, 2004, entitled METHODS OF USING (3,2,0) HETEROCYCLIC COMPOUNDS AND ANALOGS THEREOF; the disclosures of both of which are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The present invention relates to certain compounds and to methods for the preparation and the use of certain compounds in the fields of chemistry and medicine. Embodiments of the invention disclosed herein relate to methods of using heterocyclic compounds. In some embodiments, the compounds are used as proteasome inhibitors. In other embodiments, the compounds are used to treat inflammation, cancer, and infectious diseases.

[0004] 2. Description of the Related Art

[0005] Cancer is a leading cause of death in the United States. Despite significant efforts to find new approaches for treating cancer, the primary treatment options remain surgery, chemotherapy and radiation therapy, either alone or in combination. Surgery and radiation therapy, however, are generally useful only for fairly defined types of cancer, and are of limited use for treating patients with disseminated disease. Chemotherapy is the method that is generally useful in treating patients with metastatic cancer or diffuse cancers such as leukemias. Although chemotherapy can provide a therapeutic benefit, it often fails to result in cure of the disease due to the patient's cancer cells becoming resistant to the chemotherapeutic agent. Due, in part, to the likelihood of cancer cells becoming resistant to a chemotherapeutic agent, such agents are commonly used in combination to treat patients.

[0006] Similarly, infectious diseases caused, for example, by bacteria, fungi and protozoa are becoming increasingly difficult to treat and cure. For example, more and more bacteria, fungi and protozoa are developing resistance to current antibiotics and chemotherapeutic agents. Examples of such microbes include *Bacillus*, *Leishmania*, *Plasmodium* and *Trypanosoma*.

[0007] Furthermore, a growing number of diseases and medical conditions are classified as inflammatory diseases. Such diseases include conditions such as asthma to cardiovascular diseases. These diseases continue to affect larger and larger numbers of people worldwide despite new therapies and medical advances.

[0008] Therefore, a need exists for additional chemotherapeutics, anti-microbial agents, and anti-inflammatory agents to treat cancer, inflammatory diseases and infectious disease. A continuing effort is being made by individual investiga-

tors, academia and companies to identify new, potentially useful chemotherapeutic and anti-microbial agents.

[0009] Marine-derived natural products are a rich source of potential new anti-cancer agents and anti-microbial agents. The oceans are massively complex and house a diverse assemblage of microbes that occur in environments of extreme variations in pressure, salinity, and temperature. Marine microorganisms have therefore developed unique metabolic and physiological capabilities that not only ensure survival in extreme and varied habitats, but also offer the potential to produce metabolites that would not be observed from terrestrial microorganisms (Okami, Y. 1993 *J Mar Biotechnol* 1:59). Representative structural classes of such metabolites include terpenes, peptides, polyketides, and compounds with mixed biosynthetic origins. Many of these molecules have demonstrable anti-tumor, anti-bacterial, anti-fungal, anti-inflammatory or immunosuppressive activities (Bull, A. T. et al. 2000 *Microbiol Mol Biol Rev* 64:573; Cragg, G. M. & D. J. Newman 2002 *Trends Pharmacol Sci* 23:404; Kerr, R. G. & S. S. Kerr 1999 *Exp Opin Ther Patents* 9:1207; Moore, B. S. 1999 *Nat Prod Rep* 16:653; Faulkner, D. J. 2001 *Nat Prod Rep* 18:1; Mayer, A. M. & V. K. Lehmann 2001 *Anticancer Res* 21:2489), validating the utility of this source for isolating invaluable therapeutic agents. Further, the isolation of novel anti-cancer and anti-microbial agents that represent alternative mechanistic classes to those currently on the market will help to address resistance concerns, including any mechanism-based resistance that may have been engineered into pathogens for bioterrorism purposes.

SUMMARY OF THE INVENTION

[0010] The embodiments disclosed herein generally relate to chemical compounds, including heterocyclic compounds and analogs thereof. Some embodiments are directed to the use of compounds as proteasome inhibitors.

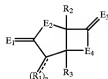
[0011] In other embodiments, the compounds are used to treat neoplastic diseases, for example, to inhibit the growth of tumors, cancers and other neoplastic tissues. The methods of treatment disclosed herein may be employed with any patient suspected of carrying tumorous growths, cancers, or other neoplastic growths, either benign or malignant ("tumor" or "tumors" as used herein encompasses tumors, cancers, disseminated neoplastic cells and localized neoplastic growths). Examples of such growths include but are not limited to breast cancers; osteosarcomas, angiosarcomas, fibrosarcomas and other sarcomas; leukemias; sinus tumors; ovarian, uretal, bladder, prostate and other genitourinary cancers; colon, esophageal and stomach cancers and other gastrointestinal cancers; lung cancers; lymphomas; myelomas; pancreatic cancers; liver cancers; kidney cancers; endocrine cancers; skin cancers; melanomas; angiomias; and brain or central nervous system (CNS) cancers. In general, the tumor or growth to be treated may be any tumor or cancer, primary or secondary. Certain embodiments relate to methods of treating neoplastic diseases in animals. The method can include, for example, administering an effective amount of a compound to a patient in need thereof. Other embodiments relate to the use of compounds in the manufacture of a pharmaceutical or medicament for the treatment of a neoplastic disease. The compounds can be administered in combination with a chemotherapeutic agent.

[0012] In still other embodiments, the compounds are used to treat inflammatory conditions. Certain embodiments

relate to methods of treating inflammatory conditions in animals. The method can include, for example, administering an effective amount of a compound to a patient in need thereof. Other embodiments relate to the use of compounds in the manufacture of a pharmaceutical or medicament for the treatment of inflammation.

[0013] In certain embodiments, the compounds are used to treat infectious diseases. The infectious agent can be a microbe, for example, bacteria, fungi, protozoans, and microscopic algae, or viruses. Further, the infectious agent can be *B. anthracis* (anthrax). In some embodiments the infectious agent is a parasite. For example, the infectious agent can be *Plasmodium*, *Leishmania*, and *Trypanosoma*. Certain embodiments relate to methods of treating infectious agents in animals. The method can include, for example, administering an effective amount of a compound to a patient in need thereof. Other embodiments relate to the use of compounds in the manufacture of a pharmaceutical or medicament for the treatment of infectious agents.

[0014] Some embodiments relate to uses of a compound having the structure of Formula I, and pharmaceutically acceptable salts and pro-drug esters thereof:



Formula I

[0015] wherein the dashed lines represent a single or a double bond, wherein R1 may be separately selected from the group consisting of a hydrogen, a halogen, mono-substituted, poly-substituted or unsubstituted variants of the following residues: saturated C₁-C₂₄ alkyl, unsaturated C₁-C₂₄ alkenyl or C₂-C₂₄ alkynyl, acyl, acyloxy, alkyl-oxycarbonyloxy, aryloxy-carbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy carbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl, where n is equal to 1 or 2, and if n is equal to 2, then R₁ can be the same or different;

[0016] wherein R₂ may be selected from the group consisting of hydrogen, a halogen, mono-substituted, poly-substituted or unsubstituted variants of the following residues: saturated C₁-C₂₄ alkyl, unsaturated C₂-C₂₄ alkenyl or C₂-C₂₄ alkynyl, acyl, acyloxy, alkyl-oxycarbonyloxy, aryloxy-carbonyloxy, cycloalkyl, cycloalkenyl (including, for example, cyclohexylcarbinol), alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy carbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

[0017] wherein R₃ may be selected from the group consisting of a halogen, mono-substituted, poly-substituted or unsubstituted variants of the following residues: saturated C₁-C₂₄ alkyl, unsaturated C₂-C₂₄ alkenyl or C₂-C₂₄ alkynyl, acyl, acyloxy, alkyl-oxycarbonyloxy, aryloxy-carbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy carbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl; and wherein each of E₁, E₂, E₃ and E₄ is a substituted or unsubstituted heteroatom; in the treatment of cancer, inflammation, and infectious disease.

[0018] Other embodiments relate to methods of treating a neoplastic disease in an animal. The methods can include, for example, administering to the animal, a therapeutically effective amount of a compound of a formula selected from Formulae I-V, and pharmaceutically acceptable salts and pro-drug esters thereof.

[0019] Further embodiments relate to pharmaceutical compositions which include a compound of a formula selected from Formulae I-V. The pharmaceutical compositions can further include an anti-microbial agent.

[0020] Still further embodiments relate to methods of inhibiting the growth of a cancer cell. The methods can include, for example, contacting a cancer cell with a compound of a formula selected from Formulae I-V, and pharmaceutically acceptable salts and pro-drug esters thereof.

[0021] Other embodiments relate to methods of inhibiting proteasome activity that include the step contacting a cell with a compound of a formula selected from Formulae I-V, and pharmaceutically acceptable salts and pro-drug esters thereof.

[0022] Other embodiments relate to methods of inhibiting NF-κB activation including the step contacting a cell with a compound of a formula selected from Formulae I-V, and pharmaceutically acceptable salts and pro-drug esters thereof.

[0023] Some embodiments relate to methods for treating an inflammatory condition, including administering an effective amount of a compound of a formula selected from Formulae I-V to a patient in need thereof.

[0024] Further embodiments relate to methods for treating a microbial illness including administering an effective amount of a compound of a formula selected from Formulae I-V to a patient in need thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] The accompanying drawings, which are incorporated in and form part of the specification, merely illustrate certain preferred embodiments of the present invention. Together with the remainder of the specification, they are

meant to serve to explain preferred modes of making certain compounds of the invention to those of skilled in the art. In the drawings:

[0026] FIG. 1 shows the chemical structure of Salinosporamide A.

[0027] FIG. 2 shows the pan-tropical distribution of the *Salinospora*. "X" denotes *Salinospora* collection sites.

[0028] FIG. 3 shows colonies of *Salinospora*.

[0029] FIG. 4 shows the typical 16S rDNA sequence of the *Salinospora*. Bars represent characteristic signature nucleotides of the *Salinospora* that separate them from their nearest relatives.

[0030] FIG. 5 shows Omuralide, a degradation product of the microbial metabolite Lactacystin. Also shown is a compound of Formula II-16, also referred to as Salinosporamide A.

[0031] FIG. 6 illustrates lethal toxin-mediated macrophage cytotoxicity. NPI-0052 represents the compound of Formula II-16.

[0032] FIG. 7 depicts the 1H NMR spectrum of a compound having structure Formula II-20.

[0033] FIG. 8 depicts the 1H NMR spectrum of a compound having structure Formula II-24C.

[0034] FIG. 9 depicts the 1H NMR spectrum of a compound having structure Formula II-19.

[0035] FIG. 10 depicts the 1H NMR spectrum of a compound having structure Formula II-2.

[0036] FIG. 11 depicts the mass spectrum of a compound having structure Formula II-2.

[0037] FIG. 12 depicts the 1H NMR spectrum of a compound having structure Formula II-3.

[0038] FIG. 13 depicts the mass spectrum of a compound having structure Formula II-3.

[0039] FIG. 14 depicts the 1H NMR spectrum of a compound having structure Formula II-4.

[0040] FIG. 15 depicts the mass spectrum of a compound having structure Formula II-4.

[0041] FIG. 16 depicts the 1H NMR spectrum of a compound having structure Formula II-5A.

[0042] FIG. 17 depicts the mass spectrum of a compound having structure Formula II-5A.

[0043] FIG. 18 depicts the 1H NMR spectrum of a compound having structure Formula II-5B.

[0044] FIG. 19 depicts the mass spectrum of a compound having structure Formula II-5B.

[0045] FIG. 20 depicts the 1H NMR spectrum of a compound having structure Formula IV-3C in DMSO-d₆.

[0046] FIG. 21 depicts the 1H NMR spectrum of a compound having structure Formula IV-3C in C₆D₆/DMSO-d₆.

[0047] FIG. 22 depicts the 1H NMR spectrum of a compound having structure Formula II-13C.

[0048] FIG. 23 depicts the 1H NMR spectrum of a compound having structure Formula II-8C.

[0049] FIG. 24 depicts the 1H NMR spectrum of a compound having structure Formula II-25.

[0050] FIG. 25 depicts the 1H NMR spectrum of a compound having structure Formula II-21.

[0051] FIG. 26 depicts the 1H NMR spectrum of a compound having structure Formula II-22.

[0052] FIG. 27 shows inhibition of the chymotrypsin-like activity of rabbit muscle proteasomes.

[0053] FIG. 28 shows inhibition of the PGPB activity of rabbit muscle proteasomes.

[0054] FIG. 29 shows inhibition of the chymotrypsin-like activity of human erythrocyte proteasomes.

[0055] FIG. 30 shows the effect of II-16 treatment on chymotrypsin-mediated cleavage of LLVY-AMC substrate.

[0056] FIG. 31 shows NF-κB/luciferase activity and cytotoxicity profiles of II-16.

[0057] FIG. 32 shows reduction of IκBα degradation and retention of phosphorylated IκBα by II-16 in HEK293 cells (A) and the HEK293 NF-κB/Luciferase reporter clone (B).

[0058] FIG. 33 shows accumulation of cell cycle regulatory proteins, p21 and p27, by II-16 treatment of HEK293 cells (A) and the HEK293 NF-κB/Luciferase reporter clone (B).

[0059] FIG. 34 shows activation of Caspase-3 by II-16 in Jurkat cells.

[0060] FIG. 35 shows PARP cleavage by II-16 in Jurkat cells.

[0061] FIG. 36 shows inhibition of LeTx-induced cytotoxicity by II-16 in RAW264.7 cells.

[0062] FIG. 37 shows the effects of II-16 treatment on PARP and Pro-Caspase 3 cleavage in RPMI 8226 and PC-3 cells.

[0063] FIG. 38 shows II-16 treatment of RPMI 8226 results in a dose-dependent cleavage of PARP and Pro-Caspase 3.

[0064] FIG. 39 shows in vitro proteasome inhibition by II-16, I-17, and II-18.

[0065] FIG. 40 shows proteasomal activity in PWBL prepared from II-16 treated mice.

[0066] FIG. 41 shows epoxomicin treatment in the PWBL assay.

[0067] FIG. 42 shows intra-assay comparison.

[0068] FIG. 43 shows decreased plasma TNF levels in mice treated with LPS.

[0069] FIG. 44 depicts assay results showing the effect of Formula II-2, Formula II-3 and Formula II-4 on NF-κB mediated luciferase activity in HEK293 NF-κB/Luc Cells.

[0070] FIG. 45 depicts assay results showing the effect of Formula II-5A and Formula II-5B on NF-κB mediated luciferase activity in HEK293 NF-κB/Luc Cells.

[0071] FIG. 46 depicts assay results showing the effect of Formula II-2, Formula II-3, and Formula II-4 on the chymotrypsin-like activity of rabbit 20S proteasome.

[0072] FIG. 47 depicts the effect of Formula II-5A and Formula II-5B on the chymotrypsin-like activity of rabbit 20S proteasome.

[0073] FIG. 48 depicts the effect of Formulas II-2, II-3, and II-4 against LeTx-mediated cytotoxicity.

[0074] FIG. 49 depicts the ¹H NMR spectrum of a compound having structure Formula II-17.

[0075] FIG. 50 depicts the ¹H NMR spectrum of a compound having structure Formula II-18.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

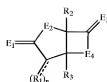
[0076] Numerous references are cited herein. The references cited herein, including the U.S. patents cited herein, are each to be considered incorporated by reference in their entirety into this specification.

[0077] Embodiments of the invention include, but are not limited to, providing a method for the preparation of compounds, including compounds, for example, those described herein and analogs thereof, and to providing a method for producing pharmaceutically acceptable anti-microbial, anti-cancer, and anti-inflammatory compositions, for example. The methods can include the compositions in relatively high yield, wherein the compounds and/or their derivatives are among the active ingredients in these compositions. Other embodiments relate to providing novel compounds not obtainable by currently available methods. Furthermore, embodiments relate to methods of treating cancer, inflammation, and infectious diseases, particularly those affecting humans. The methods may include, for example, the step of administering an effective amount of a member of a class of new compounds. Preferred embodiments relate to the compounds and methods of making and using such compounds disclosed herein, but not necessarily in all embodiments of the present invention, these objectives are met.

[0078] For the compounds described herein, each stereocenter may be of R or S configuration. Although the specific compounds exemplified in this application may be depicted in a particular configuration, compounds having either the opposite stereochemistry at any given chiral center or mixtures thereof are also envisioned. When chiral centers are found in the derivatives of this invention, it is to be understood that the compounds encompasses all possible stereoisomers.

[0079] Compounds of Formula I

[0080] Some embodiments provide compounds, and methods of producing a class of compounds, pharmaceutically acceptable salts and pro-drug esters thereof, wherein the compounds are represented by Formula I:

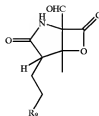


Formula I

[0081] In certain embodiments the substituent(s) R₁, R₂, and R₃ separately may include a hydrogen, a halogen, a mono-substituted, a poly-substituted or an unsubstituted variant of the following residues: saturated C₁-C₂₄ alkyl, unsaturated C₂-C₂₄ alkenyl or C₂-C₂₄ alkynyl, acyl, acyloxy, alkoxycarbonyloxy, aryloxy, aryloxy, cycloalkyl (including for example, cyclohexylcarbinol), cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy, carbonyl, alkoxy carbonyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl. Further, in certain embodiments, each of E₁, E₂, E₃ and E₄ may be a substituted or unsubstituted heteroatom, for example, a heteroatom separately selected from the group consisting of nitrogen, sulfur and oxygen.

[0082] In some embodiments n may be equal to 1 or equal to 2. When n is equal to 2, the substituents can be the same or can be different. Furthermore, in some embodiments R₃ is not a hydrogen.

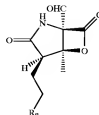
[0083] Preferably, R₂ may be a formyl. For example, the compound may have the following structure I-1:



Formula I-1

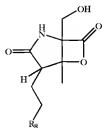
[0084] R₈ may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0085] Preferably, the structure of Formula I-1 may have the following stereochemistry:



[0086] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

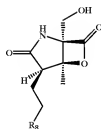
[0087] Preferably, R_2 may be a carbinol. For example, the compound may have the following structure I-2:



Formula I-2

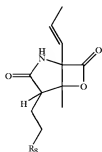
[0088] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0089] As an example, the structure of Formula I-2 may have the following stereochemistry:



[0090] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

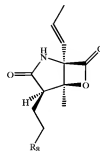
[0091] As exemplary compound of Formula I may be the compound having the following structure I-3:



Formula I-3

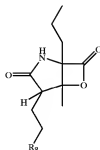
[0092] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0093] The compound of Formula I-3 may have the following stereochemical structure:



[0094] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

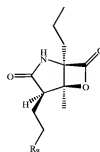
[0095] Another exemplary compound Formula I may be the compound having the following structure I-4:



Formula I-4

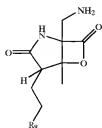
[0096] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0097] Preferably, the compound of Formula I-4 may have the following stereochemical structure:



[0098] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

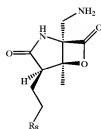
[0099] Still a further exemplary compound of Formula I is the compound having the following structure 1-5:



Formula I-5

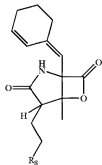
[0100] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0101] For example, the compound of Formula I-5 may have the following stereochemistry:



[0102] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

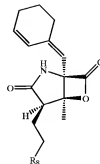
[0103] In some embodiments, R_2 of Formula I may be, for example, a 3-methylcyclohexene. For example, the compound may have the following structure of Formula I-6:



Formula I-6

[0104] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0105] Preferably, the compound of Formula I-6 may have the following stereochemistry:



[0106] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

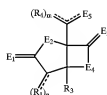
[0107] In other embodiments, R_2 may be a cyclohexylalkylamine.

[0108] Also, in other embodiments, R_2 may be a C-Cyclohexyl-methylenamine. In others, R_2 may be a cyclohexanecarbaldehyde O-oxime.

[0109] Furthermore, in some embodiments, R_2 may be a cycloalkylacyl.

[0110] Compounds of Formula II

[0111] Other embodiments provide compounds, and methods of producing a class of compounds, pharmaceutically acceptable salts and pro-drug esters thereof, wherein the compounds are represented by Formula II:



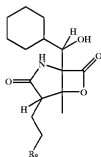
Formula II

[0112] In certain embodiments the substituent(s) R_1 , R_2 , R_3 , and R_4 separately may include a hydrogen, a halogen, a mono-substituted, a poly-substituted or an unsubstituted variant of the following residues: saturated C_1 - C_{24} alkyl, unsaturated C_2 - C_{24} alkenyl or C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy carbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxyalkyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl. Further, in certain embodiments, each of E_1 , E_2 , E_3 and E_4 may be a substituted or unsubstituted heteroatom, for example, a heteroatom or substituted heteroatom selected from the group consisting of nitrogen, sulfur and oxygen.

[0113] In some embodiments n may be equal to 1, while in others it may be equal to 2. When n is equal to 2, the substituents can be the same or can be different. Furthermore, in some embodiments R_3 is not a hydrogen. m can be equal to 1 or 2, and when m is equal to 2, R_4 can be the same or different.

[0114] E_5 may be, for example, OH, O, OR₁₀, S, SR₁₁, SO₂R₁₂, NH, NH₂, NOH, NHOH, NR₁₂, and NHOR₁₃, wherein R₁₀₋₁₃ may separately include, for example, hydrogen, a substituted or unsubstituted any of the following: alkyl, an aryl, a heteroaryl, and the like. Also, R_3 may be CH₂CH₂X, wherein X may be, for example, H, F, Cl, Br, and I. R_3 may be methyl. Furthermore, R_4 may include a cyclohexyl. Also, each of E_1 , E_3 and E_4 may be O and E_2 may be NH. Preferably, R_1 may be CH₂CH₂X, wherein X is selected from the group consisting of H, F, Cl Br, and I; wherein R_4 may include a cyclohexyl; wherein R_3 may be methyl; and wherein each of E_1 , E_3 and E_4 separately may be O and E_2 may be NH.

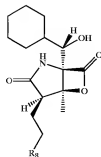
[0115] For example, an exemplary compound of Formula II has the following structure II-1:



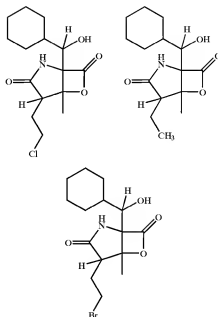
Formula II-1

[0116] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

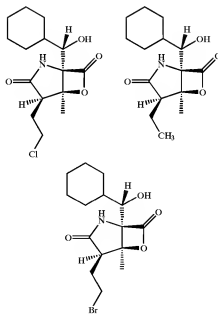
[0117] Exemplary stereochemistry may be as follows:



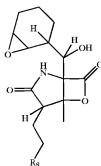
[0118] In preferred embodiments, the compound of Formula II has any of the following structures:



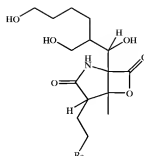
[0119] The following is exemplary stereochemistry for compounds having the structures II-2, II-3, and II-4, respectively:



[0120] In other embodiments wherein R_4 may include a 7-oxa-bicyclo[4.1.0]hept-2-yl). An exemplary compound of Formula II is the following structure II-5:



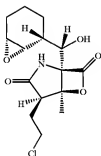
Formula II-5



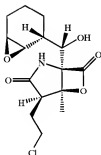
Formula II-6

[0121] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0122] The following are examples of compounds having the structure of Formula II-5:



II-5A

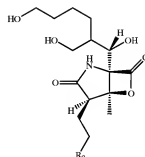


II-5B

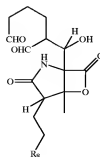
[0123] In still further embodiments, at least one R_4 may include a substituted or an unsubstituted branched alkyl. For example, a compound of Formula II may be the following structure II-6:

[0124] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0125] The following is exemplary stereochemistry for a compound having the structure of Formula II-6:



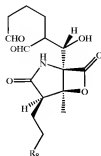
[0126] As another example, the compound of Formula II may be the following structure II-7:



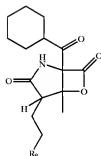
Formula II-7

[0127] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0128] The following is exemplary stereochemistry for a compound having the structure of Formula II-7:



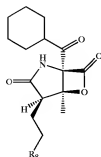
[0129] In other embodiments, at least one R_4 may be a cycloalkyl and E_3 may be an oxygen. An exemplary compound of Formula II may be the following structure II-8:



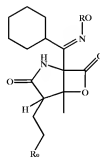
Formula II-8

[0130] R_8 may include, for example, hydrogen (II-8A), fluorine (II-8B), chlorine (II-8C), bromine (II-8D) and iodine (II-8E).

[0131] The following is exemplary stereochemistry for a compound having the structure of Formula II-8:



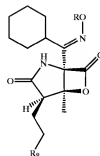
[0132] In some embodiments E_5 may be an amine oxide, giving rise to an oxime. An exemplary compound of Formula II has the following structure II-9:



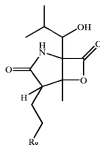
Formula II-9

[0133] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine; R may be hydrogen, and a substituted or unsubstituted alkyl, aryl, or heteroaryl, and the like.

[0134] The following is exemplary stereochemistry for a compound having the structure of Formula II-9:



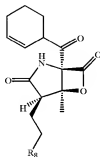
[0135] A further exemplary compound of Formula II has the following structure II-10:



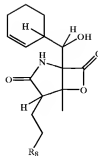
Formula II-10

[0136] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

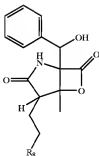
[0146] The following is exemplary stereochemistry for a compound having the structure of Formula II-13 :



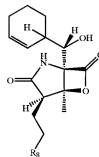
Formula II-13



[0147] A still further exemplary compound of Formula II has the following structure II-14:

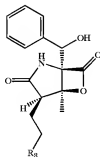


Formula II-14



[0148] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0149] The following is exemplary stereochemistry for a compound having the structure of Formula II-14:



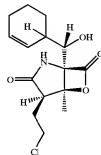
[0150] In some embodiments, the compounds of Formula II, may include as R_4 at least one cycloalkene, for example. Furthermore, in some embodiments, the compounds may include a hydroxy at E_5 , for example. A further exemplary compound of Formula II has the following structure II-15:

[0151] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

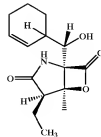
[0152] Exemplary stereochemistry may be as follows:

[0153] The following is exemplary stereochemistry for compounds having the structures II-16, II-17, II-18, and II-19, respectively:

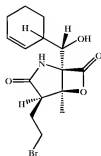
II-16



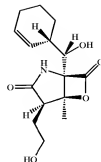
II-17



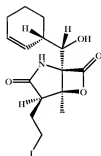
-continued



II-18

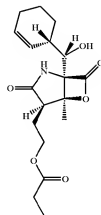


II-21



II-19

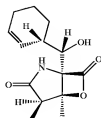
[0157] In some embodiments, the hydroxyl group of Formula II-21 may be esterified such that R_1 may include ethylpropionate, for example. An exemplary compound, Formula II-22, has the following structure and stereochemistry:



II-22

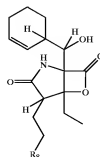
[0154] The compounds of Formulae II-16, II-17, II-18 and II-19 may be obtained by fermentation, synthesis, or semi-synthesis and isolated/purified as set forth below. Furthermore, the compounds of Formulae II-16, II-17, II-18 and II-19 may be used, and are referred to, as “starting materials” to make other compounds described herein.

[0155] In some embodiments, the compounds of Formula II, may include a methyl group as R_1 , for example. A further exemplary compound, Formula II-20, has the following structure and stereochemistry:



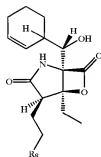
II-20

[0158] In some embodiments, the compounds of Formula II may include an ethyl group as R_3 , for example. A further exemplary compound of Formula II has the following structure II-23:



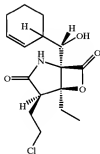
[0156] In some embodiments, the compounds of Formula II, may include hydroxyethyl as R_1 , for example. A further exemplary compound, Formula II-21, has the following structure and stereochemistry:

[0159] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine. Exemplary stereochemistry may be as follows:



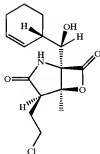
[0160] In some embodiments, the compounds of Formula II-23 may have the following structure and stereochemistry, exemplified by Formula II-24C, where R_8 is chlorine:

II-24C



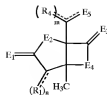
[0161] In some embodiments, the compounds of Formula II-15 may have the following stereochemistry, exemplified by the compound of Formula II-25, where R_8 is chlorine:

II-25



[0162] Compounds of Formula III

[0163] Other embodiments provide compounds, and methods of producing a class of compounds, pharmaceutically acceptable salts and pro-drug esters thereof, wherein the compounds are represented by Formula III:



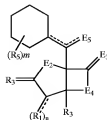
Formula III

[0164] In certain embodiments, the substituent(s) R_1 separately may include, for example, a hydrogen, a halogen, a mono-substituted, a poly-substituted or an unsubstituted variant of the following residues: saturated C_1 - C_{24} alkyl, unsaturated C_2 - C_{24} alkenyl or C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy carbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl. For example, n can be equal to 1 or 2.

[0165] In certain embodiments, R_1 may be, for example, a hydrogen, a halogen, a mono-substituted, a poly-substituted or an unsubstituted variants of the following residues: saturated C_1 - C_{24} alkyl, unsaturated C_2 - C_{24} alkenyl or C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy carbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl. In some embodiments m can be equal to 1 or 2, and where m is equal to 2, the substituents can be the same or different. Also, each of E_1 , E_2 , E_3 , E_4 and E_5 may be, for example, a substituted or unsubstituted heteroatom. For example, the heteroatom may be nitrogen, sulfur or oxygen.

[0166] Compounds of Formula IV

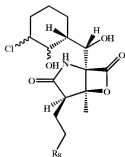
[0167] Other embodiments provide compounds, and methods of producing a class of compounds, pharmaceutically acceptable salts and pro-drug esters thereof, wherein the compounds are represented by Formula IV:



Formula IV

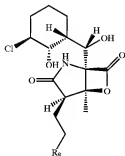
[0168] In certain embodiments, the substituent(s) R_1 , R_3 , and R_5 may separately include a hydrogen, a halogen, a

[0176] Exemplary structure and stereochemistry may be as follows:



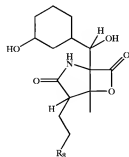
Formula IV-3C

[0177] Additional exemplary structure and stereochemistry may be as follows:



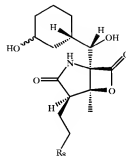
Formula IV-4

[0178] For example, an exemplary compound of Formula IV has the following structure IV-4:



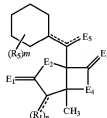
[0179] R₈ may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0180] Exemplary stereochemistry may be as follows:



[0181] Compounds of Formula V

[0182] Some embodiments provide compounds, and methods of producing a class of compounds, pharmaceutically acceptable salts and pro-drug esters thereof, wherein the compounds are represented by Formula V:



Formula V

[0183] In certain embodiments, the substituent(s) R₁ and R₂ may separately include a hydrogen, a halogen, a mono-substituted, a poly-substituted or unsubstituted variants of the following residues: saturated C₁-C₂₄ alkyl, unsaturated C₃-C₂₄ alkenyl or C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxy-carbonyloxy, aryloxy-carbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy carbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl. In certain embodiments, each of E₁, E₂, E₃, E₄ and E₅ may be a heteroatom or substituted heteroatom, for example, nitrogen, sulfur or oxygen. n can be equal to 1 or 2, and when n is equal to 2, the substituents can be the same or different. Preferably, m may be, for example, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11. When m is greater than 1, R₅ may be the same or different.

[0184] Certain embodiments also provide pharmaceutically acceptable salts and pro-drug esters of the compound of Formulae I-V, and provide methods of obtaining and purifying such compounds by the methods disclosed herein.

[0185] The term "pro-drug ester," especially when referring to a pro-drug ester of the compound of Formula I synthesized by the methods disclosed herein, refers to a chemical derivative of the compound that is rapidly transformed in vivo to yield the compound, for example, by hydrolysis in blood or inside tissues. The term "pro-drug ester" refers to derivatives of the compounds disclosed herein formed by the addition of any of several ester-forming groups that are hydrolyzed under physiological conditions. Examples of pro-drug ester groups include pivaloxymethyl, acetoxymethyl, phthalidyl, indanyl and methoxymethyl, as well as other such groups known in the art, including a (5-R-2-oxo-1,3-dioxolen-4-yl)methyl group. Other examples of pro-drug ester groups can be found, for example, T. Higuchi and V. Stella, in "Pro-drugs as Novel Delivery Systems", Vol. 14, A.C.S. Symposium Series, American Chemical Society (1975); and "Bioreversible Carriers in Drug Design: Theory and Application", edited by E. B. Roche, Pergamon Press: New York, 14-21 (1987) (providing examples of esters useful as prodrugs for compounds containing carboxyl groups). Each of the above-mentioned references is hereby incorporated by reference in its entirety.

[0186] The term "pro-drug ester," as used herein, also refers to a chemical derivative of the compound that is rapidly transformed in vivo to yield the compound, for example, by hydrolysis in blood.

[0187] The term "pharmaceutically acceptable salt," as used herein, and particularly when referring to a pharmaceutically acceptable salt of a compound, including Formulae I-V, and Formula I-V as produced and synthesized by the methods disclosed herein, refers to any pharmaceutically acceptable salts of a compound, and preferably refers to an acid addition salt of a compound. Preferred examples of pharmaceutically acceptable salt are the alkali metal salts (sodium or potassium), the alkaline earth metal salts (calcium or magnesium), or ammonium salts derived from ammonia or from pharmaceutically acceptable organic amines, for example C₁-C₇ alkylamine, cyclohexylamine, triethanolamine, ethylenediamine or tris(hydroxymethyl)aminomethane. With respect to compounds synthesized by the method of this embodiment that are basic amines, the preferred examples of pharmaceutically acceptable salts are acid addition salts of pharmaceutically acceptable inorganic or organic acids, for example, hydrohalic, sulfuric, phosphoric acid or aliphatic or aromatic carboxylic or sulfonic acid, for example acetic, succinic, lactic, malic, tartaric, citric, ascorbic, nicotinic, methanesulfonic, p-toluenesulfonic or naphthalenesulfonic acid.

[0188] Preferred pharmaceutical compositions disclosed herein include pharmaceutically acceptable salts and pro-drug esters of the compound of Formulae I-V obtained and purified by the methods disclosed herein. Accordingly, if the manufacture of pharmaceutical formulations involves intimate mixing of the pharmaceutical excipients and the active ingredient in its salt form, then it is preferred to use pharmaceutical excipients which are non-basic, that is, either acidic or neutral excipients.

[0189] It will be also appreciated that the phrase "compounds and compositions comprising the compound," or any like phrase, is meant to encompass compounds in any suitable form for pharmaceutical delivery, as discussed in further detail herein. For example, in certain embodiments,

the compounds or compositions comprising the same may include a pharmaceutically acceptable salt of the compound.

[0190] In one embodiment the compounds may be used to treat microbial diseases, cancer, and inflammation. Disease is meant to be construed broadly to cover infectious diseases, and also autoimmune diseases, non-infectious diseases and chronic conditions. In a preferred embodiment, the disease is caused by a microbe, such as a bacterium, a fungi, and protozoa, for example. The methods of use may also include the steps of administering a compound or composition comprising the compound to an individual with an infectious disease or cancer. The compound or composition can be administered in an amount effective to treat the particular infectious disease, cancer or inflammatory condition.

[0191] The infectious disease may be, for example, one caused by *Bacillus*, such as *B. anthracis* and *B. cereus*. The infectious disease may be one caused by a protozoa, for example, a *Leishmania*, a *Plasmodium* or a *Trypanosoma*. The compound or composition may be administered with a pharmaceutically acceptable carrier, diluent, excipient, and the like.

[0192] The cancer may be, for example, a multiple myeloma, a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma, a melanoma, and the like.

[0193] The inflammatory condition may be, for example, rheumatoid arthritis, asthma, multiple sclerosis, psoriasis, stroke, myocardial infarction, and the like.

[0194] The term "halogen atom," as used herein, means any one of the radio-stable atoms of column 7 of the Periodic Table of the Elements, i.e., fluorine, chlorine, bromine, or iodine, with bromine and chlorine being preferred.

[0195] The term "alkyl," as used herein, means any unbranched or branched, substituted or unsubstituted, saturated hydrocarbon, with C₁-C₆ unbranched, saturated, unsubstituted hydrocarbons being preferred, with methyl, ethyl, isobutyl, and tert-butylpropyl, and pentyl being most preferred. Among the substituted, saturated hydrocarbons, C-C₆ mono- and di- and per-halogen substituted saturated hydrocarbons and amino-substituted hydrocarbons are preferred, with perfluoromethyl, perchloromethyl, perfluoro-tert-butyl, and perchloro-tert-butyl being the most preferred.

[0196] The term "substituted" has its ordinary meaning, as found in numerous contemporary patents from the related art. See, for example, U.S. Pat. Nos. 6,509,331; 6,506,787; 6,500,825; 5,922,683; 5,886,210; 5,874,443; and 6,350,759; all of which are incorporated herein in their entireties by reference. Specifically, the definition of substituted is as broad as that provided in U.S. Pat. No. 6,509,331, which defines the term "substituted alkyl" such that it refers to an alkyl group, preferably of from 1 to 10 carbon atoms, having from 1 to 5 substituents, and preferably 1 to 3 substituents, selected from the group consisting of alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, acyl, acylamino, acyloxy, amino, substituted amino, aminoacyl, aminoacyloxy, oxyacylamino, cyano, halogen, hydroxyl, carboxyl, carboxyalkyl, keto, thioketo, thiol, thioalkoxy, substituted thioalkoxy, aryl, aryloxy, heteroaryl, heteroaryloxy, heterocyclic, heterocycloxy, hydroxyamino, alkoxyamino, nitro, —SO-alkyl,

—SO-substituted alkyl, —SO-aryl, —SO-heteroaryl, —SO₂-alkyl, —SO₂-substituted alkyl, —SO₂-aryl and —SO₂-heteroaryl. The other above-listed patents also provide standard definitions for the term “substituted” that are well-understood by those of skill in the art.

[0197] The term “cycloalkyl” refers to any non-aromatic hydrocarbon ring, preferably having five to twelve atoms comprising the ring. The term “acyl” refers to alkyl or aryl groups derived from an oxoacid, with an acetyl group being preferred.

[0198] The term “alkenyl,” as used herein, means any unbranched or branched, substituted or unsubstituted, unsaturated hydrocarbon including polyunsaturated hydrocarbons, with C₃-C₆ unbranched, mono-unsaturated and di-unsaturated, unsubstituted hydrocarbons being preferred, and mono-unsaturated, di-halogen substituted hydrocarbons being most preferred. The term “cycloalkenyl” refers to any non-aromatic hydrocarbon ring, preferably having five to twelve atoms comprising the ring.

[0199] The terms “aryl,” “substituted aryl,” “heteroaryl,” and “substituted heteroaryl,” as used herein, refer to aromatic hydrocarbon rings, preferably having five, six, or seven atoms, and most preferably having six atoms comprising the ring. “Heteroaryl” and “substituted heteroaryl,” refer to aromatic hydrocarbon rings in which at least one heteroatom, e.g., oxygen, sulfur, or nitrogen atom, is in the ring along with at least one carbon atom. The term “heterocycle” or “heterocyclic” refer to any cyclic compound containing one or more heteroatoms. The substituted aryls, heterocycles and heteroaryls can be substituted with any substituent, including those described above and those known in the art.

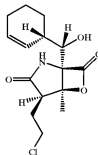
[0200] The term “alkoxy” refers to any unbranched, or branched, substituted or unsubstituted, saturated or unsaturated ether, with C₁-C₆ unbranched, saturated, unsubstituted ethers being preferred, with methoxy being preferred, and also with dimethyl, diethyl, methyl-isobutyl, and methyl-tert-butyl ethers also being preferred. The term “cycloalkoxy” refers to any non-aromatic hydrocarbon ring, preferably having five to twelve atoms comprising the ring. The term “alkoxy carbonyl” refers to any linear, branched, cyclic, saturated, unsaturated, aliphatic or aromatic alkoxy attached to a carbonyl group. The examples include methoxycarbonyl group, ethoxycarbonyl group, propyloxycarbonyl group, isopropyloxycarbonyl group, butyloxycarbonyl group, sec-butyloxycarbonyl group, tert-butyloxycarbonyl group, cyclopentyloxycarbonyl group, cyclohexyloxycarbonyl group, benzyloxycarbonyl group, allyloxycarbonyl group, phenyloxycarbonyl group, pyridyloxycarbonyl group, and the like.

[0201] The terms “pure,” “purified,” “substantially purified,” and “isolated” as used herein refer to the compound of the embodiment being free of other, dissimilar compounds with which the compound, if found in its natural state, would be associated in its natural state. In certain embodiments described as “pure,” “purified,” “substantially purified,” or “isolated” herein, the compound may comprise at least 0.5%, 1%, 5%, 10%, or 20%, and most preferably at least 50% or 75% of the mass, by weight, of a given sample.

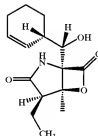
[0202] The terms “derivative,” “variant,” or other similar term refers to a compound that is an analog of the other compound.

[0203] Certain of the compounds of Formula I-V may be obtained and purified or may be obtained via semi-synthesis from purified compounds as set forth herein. Generally, without being limited thereto, the compounds of Formula II-15, preferably, Formulae II-16, II-17, II-18 and II-19, may be obtained synthetically or by fermentation. Exemplary fermentation procedures are provided below. Further, the compounds of Formula II-15, preferably, Formulae II-16, II-17, II-18 and II-19 may be used as starting compounds in order to obtain/synthesize various of the other compounds described herein. Exemplary non-limiting syntheses are provided herein.

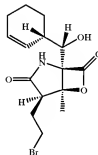
Formula II-16



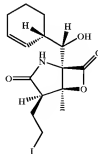
Formula II-17



Formula II-18



Formula II-19



[0204] Formula II-16 is currently produced through a high-yield saline fermentation (~200 mg/L) and modifications of the conditions has yielded new analogs in the fermentation extracts. FIG. 1 shows the chemical structure of II-16. Additional analogs can be generated through directed biosynthesis. Directed biosynthesis is the modification of a natural product by adding biosynthetic precursor analogs to the fermentation of producing microorganisms (Lam, et al., *J Antibiot (Tokyo)* 44:934 (1991); Lam, et al., *J Antibiot (Tokyo)* 54:1 (2001); which is hereby incorporated by reference in its entirety).

[0205] Exposing the producing culture to analogs of acetic acid, phenylalanine, valine, butyric acid, shikimic acid, and halogens, preferably, other than chlorine, can lead to the formation of new analogs. The new analogs produced can be easily detected in crude extracts by HPLC and LC-MS. For example, after manipulating the medium with different concentrations of sodium bromide, a bromo-analog, Formula II-18, was successfully produced in shake-flask culture at a titer of 14 mg/L.

[0206] A second approach to generate new analogs is through biotransformation. Biotransformation reactions are chemical reactions catalyzed by enzymes or whole cells containing these enzymes. Zaks, A., *Curr Opin Chem Biol* 5:130 (2001). Microbial natural products are ideal substrates for biotransformation reactions as they are synthesized by a series of enzymatic reactions inside microbial cells. Riva, S., *Curr Opin Chem Biol* 5:106 (2001).

[0207] Given the structure of the described compounds, including those of Formula II-15, for example, the possible biosynthetic origins are acetyl-CoA, ethylmalonyl-CoA, phenylalanine and chorine. Ethylmalonyl-CoA is derived from butyryl-CoA, which can be derived either from valine or crotonyl-CoA. Liu, et al., *Metab Eng* 3:40 (2001). Phenylalanine is derived from shikimic acid.

[0208] Production of Compounds of Formulae II-16, II-17, and II-18

[0209] The production of compounds of Formulae II-16, II-17, and II-18 may be carried out by cultivating strain CNB476 in a suitable nutrient medium under conditions described herein, preferably under submerged aerobic conditions, until a substantial amount of compounds are detected in the fermentation; harvesting by extracting the active components from the fermentation broth with a suitable solvent; concentrating the solvent containing the desired components; then subjecting the concentrated material to chromatographic separation to isolate the compounds from other metabolites also present in the cultivation medium.

[0210] FIG. 2 shows some collection sites worldwide for the culture (CNB476), which is also referred to as *Salinospira*. FIG. 3 shows colonies of *Salinospira*. FIG. 4 shows the typical 16S rDNA sequence of the *Salinospira*. Bars represent characteristic signature nucleotides of the *Salinospira* that separate them from their nearest relatives.

[0211] The culture (CNB476) was deposited on Jun. 20, 2003 with the American Type Culture Collection (ATCC) in Rockville, Md. and assigned the ATCC patent deposit number PTA-5275. The ATCC deposit meets all of the requirements of the Budapest treaty. The culture is also maintained at and available from Nereus Pharmaceutical

Culture Collection at 10480 Wateridge Circle, San Diego, Calif. 92121. In addition to the specific microorganism described herein, it should be understood that mutants, such as those produced by the use of chemical or physical mutagens including X-rays, etc. and organisms whose genetic makeup has been modified by molecular biology techniques, may also be cultivated to produce the starting compounds of Formulae II-16, II-17, and II-18.

[0212] Fermentation of Strain CNB476

[0213] Production of compounds can be achieved at temperature conducive to satisfactory growth of the producing organism, e.g. from 16 degree C. to 40 degree C., but it is preferable to conduct the fermentation at 22 degree C. to 32 degree C. The aqueous medium can be incubated for a period of time necessary to complete the production of compounds as monitored by high pressure liquid chromatography (HPLC), preferably for a period of about 2 to 10 days, on a rotary shaker operating at about 50 rpm to 400 rpm, preferably at 150 rpm to 250 rpm, for example.

[0214] Growth of the microorganisms may be achieved by one of ordinary skill of the art by the use of appropriate medium. Broadly, the sources of carbon include glucose, fructose, mannose, maliose, galactose, mannitol and glycerol, other sugars and sugar alcohols, starches and other carbohydrates, or carbohydrate derivatives such as dextran, cerelose, as well as complex nutrients such as oat flour, corn meal, millet, corn, and the like. The exact quantity of the carbon source that is utilized in the medium will depend in part, upon the other ingredients in the medium, but an amount of carbohydrate between 0.5 to 25 percent by weight of the medium can be satisfactorily used, for example. These carbon sources can be used individually or several such carbon sources may be combined in the same medium, for example. Certain carbon sources are preferred as hereinafter set forth.

[0215] The sources of nitrogen include amino acids such as glycine, arginine, threonine, methionine and the like, ammonium salt, as well as complex sources such as yeast extracts, corn steep liquors, distiller solubles, soybean meal, cottonseed meal, fish meal, peptone, and the like. The various sources of nitrogen can be used alone or in combination in amounts ranging from 0.5 to 25 percent by weight of the medium, for example.

[0216] Among the nutrient inorganic salts, which can be incorporated in the culture media, are the customary salts capable of yielding sodium, potassium, magnesium, calcium, phosphate, sulfate, chloride, carbonate, and like ions. Also included are trace metals such as cobalt, manganese, iron, molybdenum, zinc, cadmium, and the like.

[0217] Biological Activity and Uses of Compounds

[0218] Some embodiments relate to methods of treating cancer, inflammation, and infectious diseases, particularly those affecting humans. The methods may include, for example, the step of administering an effective amount of a member of a class of new compounds. Thus, the compounds disclosed herein may be used to treat cancer, inflammation, and infectious disease.

[0219] The compounds have various biological activities. For example, the compounds have chemosensitizing activity, anti-microbial, anti-inflammation, and anti-cancer activity.

[0220] The compounds have proteasome inhibitory activity. The proteasome inhibitory activity may, in whole or in part, contribute to the ability of the compounds to act as anti-cancer, anti-inflammatory, and anti-microbial agents.

[0221] The proteasome is a multisubunit protease that degrades intracellular proteins through its chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolyzing (PGPH); and also known as the caspase-like activity) activities. The 26S proteasome contains a proteolytic core called the 20S proteasome and two 19S regulatory subunits. The 20S proteasome is responsible for the proteolytic activity against many substrates including damaged proteins, the transcription factor NF- κ B and its inhibitor I κ B, signaling molecules, tumor suppressors and cell cycle regulators. There are three distinct protease activities within the proteasome: 1) chymotrypsin-like; 2) trypsin-like; and the 3) peptidyl glutamyl peptide hydrolyzing (PGPH) activity.

[0222] As an example, compounds of Formula II-16 were more potent (EC_{50} 2 nM) at inhibiting the chymotrypsin-like activity of rabbit muscle proteasomes than Omuralide (EC_{50} 52 nM) and also inhibited the chymotrypsin-like activity of human erythrocyte derived proteasomes (EC_{50} ~250 pM). FIG. 5 shows omuralide, which is a degradation product of Lactacystin, and it shows a compound of Formula II-16. Compounds of Formula II-16 exhibit a significant preference for inhibiting chymotrypsin-like activity of the proteasome over inhibiting the catalytic activity of chymotrypsin. Compounds of Formula II-16 also exhibit low nM trypsin-like inhibitory activity (~10 nM), but are less potent at inhibiting the PGPH activity of the proteasome (EC_{50} ~350 nM).

[0223] Additional studies have characterized the effects of compounds described herein, including studies of Formula II-16 on the NF- κ B/I κ B signaling pathway. Treatment of HEK293 cells (human embryonic kidney) with Tumor Necrosis Factor- α (TNF- α) induces phosphorylation and proteasome-mediated degradation of I κ B α : followed by NF- κ B activation. To confirm proteasome inhibition, HEK293 cells were pre-treated for 1 hour with compounds of Formula II-16 followed by TNF- α stimulation. Treatment with compounds of Formula II-16 promoted the accumulation of phosphorylated I κ B α suggesting that the proteasome-mediated I κ B α degradation was inhibited.

[0224] Furthermore, a stable HEK293 clone (NF- κ B/Luc 293) was generated carrying a luciferase reporter gene under the regulation of 5x NF- κ B binding sites. Stimulation of NF- κ B/Luc 293 cells with TNF- α increases luciferase activity as a result of NF- κ B activation while pretreatment with compounds of Formula II-16 decreases activity. Western blot analyses demonstrated that compounds of Formula II-16 promoted the accumulation of phosphorylated-I κ B α and decreased the degradation of total I κ B α in the NF- κ B/Luc 293 cells. Compounds of Formula II-16 were also shown to increase the levels of the cell cycle regulatory proteins, p21 and p27.

[0225] Tumor cells may be more sensitive to proteasome inhibitors than normal cells. Moreover, proteasome inhibition increases the sensitivity of cancer cells to anticancer agents. The cytotoxic activity of the compounds described herein, including Formula II-16, were examined for cytotoxic activity against various cancer cell lines. Formula II-16 was examined, for example, in the National Cancer Institute

screen of 60 human tumor cell lines. Formula II-16 exhibited selective cytotoxic activity with a mean GI_{50} value (the concentration to achieve 50% growth inhibition) of less than 10 nM. The greatest potency was observed against SK-MEL-28 melanoma and MDA-MB-235 breast cancer cells [both with LC_{50} (the concentration with 50% cell lethality) <10 nM].

[0226] A panel of cell lines including human colorectal (HT-29 and LoVo), prostate (PC3), breast (MDA-MB-231), lung (NCI-H292), ovarian (OVCAR3), acute T-cell leukemia (Jurkat), murine melanoma (B16-F10) and normal human fibroblasts (CCD-27sk) was treated with Salinosporamide A for 48h to assess cytotoxic activity. HT-29, LoVo, PC3, MDA-MB-231, NCI-H292, OVCAR3, Jurkat, and B16-F10 cells were sensitive with EC_{50} values of 47, 69, 78, 67, 97, 69, 10, and 33 nM, respectively. In contrast, the EC_{50} values for CCD-27sk cells were 196 nM. Treatment of Jurkat cells with Salinosporamide A at the approximate EC_{50} resulted in Caspase-3 activation and cleavage of PARP confirming the induction of apoptosis.

[0227] The anti-anthrax activity of the described compounds was evaluated using an in vitro LeTx induced cytotoxicity assay. As one example, the results indicate that Formula II-16 is a potent inhibitor of LeTx-induced cytotoxicity of murine macrophage-like RAW264.7 cells. Treatment of RAW264.7 cells with Formula II-16 resulted in a 10-fold increase in the viability of LeTx-treated cells compared to LeTx treatment alone (average EC_{50} of <4 nM).

[0228] Potential Chemosensitizing Effects of Formula II-16

[0229] Additional studies have characterized the effects of the compounds described herein on the NF- κ B/I κ B signaling pathway (see the Examples). In unstimulated cells, the transcription factor nuclear factor-kappa B (NF- κ B) resides in the cytoplasm in an inactive complex with the inhibitory protein I κ B (inhibitor of NF- κ B). Various stimuli can cause I κ B phosphorylation by I κ B kinase, followed by ubiquitination and degradation by the proteasome. Following the degradation of I κ B, NF- κ B translocates to the nucleus and regulates gene expression, affecting many cellular processes including inhibition of apoptosis. Chemotherapy agents such as CPT-11 (Irinotecan) can activate NF- κ B in human colon cancer cell lines including LoVo cells, resulting in a decreased ability of these cells to undergo apoptosis. Painter, R. B. *Cancer Res* 38:4445 (1978). Velcade™ is a dipeptidyl boronic acid that inhibits the chymotrypsin-like activity of the proteasome (Lightcap, et al., *Clin Chem* 46:673 (2000), Adams, et al., *Cancer Res* 59:2615 (1999), Adams, *Curr Opin Oncol* 14:628 (2002)) while enhancing the trypsin and PGPH activities. Recently approved as a proteasome inhibitor, Velcade™ (PS-341; Millennium Pharmaceuticals, Inc.) has been shown to be directly toxic to cancer cells and also enhance the cytotoxic activity of CPT-11 in LoVo cells in vitro and in a LoVo xenograft model by inhibiting I κ B degradation by the proteasome. Blum, et al., *Ann Intern Med* 80:249 (1974). In addition, Velcade™ was found to inhibit the expression of proangiogenic chemokines/cytokines Growth Related Oncogene-alpha (GRO- α) and Vascular Endothelial Growth Factor (VEGF) in squamous cell carcinoma, presumably through inhibition of the NF- κ B pathway. Dick, et al., *J Biol Chem* 271:7273

(1996). These data suggest that proteasome inhibition may not only decrease tumor cell survival and growth, but also angiogenesis.

[0230] Anti-Anthrax Activity

[0231] Another potential application for proteasome inhibitors comes from recent studies on the biodefense Category A agent *B. anthracis* (anthrax). Anthrax spores are inhaled and lodge in the lungs where they are ingested by macrophages. Within the macrophage, spores germinate, the organism replicates, resulting ultimately in killing of the cell. Before killing occurs, however, infected macrophages migrate to the lymph nodes where, upon death, they release their contents allowing the organism to enter the bloodstream, further replicate, and secrete lethal toxins. Hanna, et al., *Proc Natl Acad Sci U S A* 90:10198 (1993). Anthrax toxins are responsible for the symptoms associated with anthrax. Two proteins that play a key role in the pathogenesis of anthrax are protective antigen (PA, 83 kDa) and lethal factor (LF, 90 kDa) which are collectively known as lethal toxin (LeTx). LF has an enzymatic function, but requires PA to achieve its biological effect. Neither PA or LF cause death individually; however, when combined they cause death when injected intravenously in animals. Kalns, et al., *Biochem Biophys Res Commun* 297:506 (2002), Kalns, et al., *Biochem Biophys Res Commun* 292:41 (2002).

[0232] Protective antigen, the receptor-binding component of anthrax toxin, is responsible for transporting lethal factor into the host cell. PA oligomerizes into a ring-shaped heptamer (see FIG. 6). Each heptamer, bound to its receptor on the surface of a cell, has the ability to bind up to three molecules of LF. The complex formed between the PA heptamer and LF is taken into the cell by receptor-mediated endocytosis. Following endocytosis, LF is released into the cytosol where it attacks various cellular targets. Mogridge, et al., *Biochemistry* 41:1079 (2002), Lacy, et al., *J Biol Chem* 277:3006 (2002), Bradley, et al., *Nature* 414:225 (2001).

[0233] Lethal factor is a zinc dependent metalloprotease, which in the cytosol can cleave and inactivate signaling proteins of the mitogen-activated protein kinase kinase family (MAPKK). Duesbery, et al., *Science* 280:734 (1998), Bodart, et al., *Cell Cycle* 1:10 (2002), Vitale, et al., *J Appl Microbiol* 87:288 (1999), Vitale, et al., *Biochem J* 352 Pt 3:739 (2000). Of the seven different known MAPK kinases, six have been shown to be cleaved by LF. Within the cell, MAPK kinase pathways transduce various signals involved in cell death, proliferation, and differentiation making these proteins highly significant targets. However, certain inhibitors that prevent LeTx-induced cell death, do not prevent MAPKK cleavage by LF suggesting that this activity is not sufficient for induction of cell death. Kim, et al., *J Biol Chem* 278:7413 (2003), Lin, et al., *Curr Microbiol* 33:224 (1996).

[0234] Studies have suggested that inhibition of the proteasome can prevent LeTx-induced cell death. Tang, et al., *Infect Immun* 67:3055 (1999). Data have shown that proteasome activity is required for LeTx-mediated killing of RAW264.7 macrophage-like cells and that proteasome inhibitors protect RAW264.7 cells from LeTx. Proteasome inhibition did not block MEK1 cleavage, suggesting the LeTx pathway is not blocked upstream of MEK1 cleavage in these studies. Additionally, there is no increase in proteasome activity in cells treated with LeTx. These data suggested that a novel, potent proteasome inhibitor like the

compounds described herein, may also prevent LeTx-induced cell death as illustrated in FIG. 6.

[0235] The receptor for PA has been identified and is expressed by many cell types. Escuyer, et al., *Infect Immun* 59:3381 (1991). Lethal toxin is active in a few cell culture lines of macrophages causing cell death within a few hours. Hanna, et al., *Proc Natl Acad Sci USA* 90:10198 (1993), Kim, et al., *J Biol Chem* 278:7413 (2003), Lin, et al., *Curr Microbiol* 33:224 (1996). LeTx can induce both necrosis and apoptosis in mouse macrophage-like RAW264.7 and J774A.1 cells upon in vitro treatment.

[0236] The results indicate that the compounds described herein act as a potent inhibitor of LeTx-induced cytotoxicity of murine macrophage-like RAW264.7 cells. Treatment of RAW264.7 cells with, for example, compounds of Formula II-16, resulted in a 10-fold increase in the viability of LeTx-treated cells compared to LeTx treatment alone (average EC₅₀ of <4 nM) and therefore provide a valuable therapy for anthrax infections. Formula II-16, for example, promoted survival of RAW264.7 macrophage-like cells in the presence of LeTx indicating that this compound and its derivatives provide a valuable clinical therapeutic for anthrax infection.

[0237] Pharmaceutical Compositions

[0238] In one embodiment, the compounds disclosed herein are used in pharmaceutical compositions. The compounds preferably can be produced by the methods disclosed herein. The compounds can be used, for example, in pharmaceutical compositions comprising a pharmaceutically acceptable carrier prepared for storage and subsequent administration. Also, embodiments relate to a pharmaceutically effective amount of the products and compounds disclosed above in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985), which is incorporated herein by reference in its entirety. Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, ascorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. In addition, antioxidants and suspending agents may be used.

[0239] The compositions, particularly those of Formulas I-V, may be formulated and used as tablets, capsules, or elixirs for oral administration; suppositories for rectal administration; sterile solutions, suspensions for injectable administration; patches for transdermal administration, and sub-dermal deposits and the like. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (for example, liposomes), may be utilized.

[0240] Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds

in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or other organic oils such as soybean, grapefruit or almond oils, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[0241] Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginate acid or a salt thereof such as sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carboxypol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyes/stuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carboxypol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyes/stuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. Such formulations can be made using methods known in the art (see, for example, U.S. Pat. No. 5,733,888 (injectable compositions); U.S. Pat. No. 5,726,181 (poorly water soluble compounds); U.S. Pat. No. 5,707,641 (therapeutically active proteins or peptides); U.S. Pat. No. 5,667,809 (lipophilic agents); U.S. Pat. No. 5,576,012 (solubilizing polymeric agents); U.S. Pat. No. 5,707,615 (anti-viral formulations); U.S. Pat. No. 5,683,676 (particulate medicaments); U.S. Pat. No. 5,654,286 (topical formulations); U.S. Pat. No. 5,688,529 (oral suspensions); U.S. Pat. No. 5,445,829 (extended release formulations); U.S. Pat. No. 5,653,987 (liquid formulations); U.S. Pat. No. 5,641,515 (controlled release formulations) and U.S. Pat. No. 5,601,845 (spheroid formulations); all of which are incorporated herein by reference in their entireties.

[0242] Further disclosed herein are various pharmaceutical compositions well known in the pharmaceutical art for uses that include intraocular, intranasal, and intraauricular delivery. Pharmaceutical formulations include aqueous ophthalmic solutions of the active compounds in water-soluble form, such as ceydrops, or in gellan gum (Shedden et al., *Clin. Ther.*, 23(3):440-50 (2001)) or hydrogels (Mayer et al., *Ophthalmologica*, 210(2):101-3 (1996)); ophthalmic oint-

ments; ophthalmic suspensions, such as microparticulates, drug-containing small polymeric particles that are suspended in a liquid carrier medium (Joshi, A. 1994 *J. Ocul Pharmacol* 10:29-45); lipid-soluble formulations (Alm et al., *Prog. Clin. Biol. Res.*, 312:447-58 (1989)); and microspheres (Mordenti, *Toxicol. Sci.*, 52(1):101-6 (1999)); and ocular inserts. All of the above-mentioned references, are incorporated herein by reference in their entireties. Such suitable pharmaceutical formulations are most often and preferably formulated to be sterile, isotonic and buffered for stability and comfort. Pharmaceutical compositions may also include drops and sprays often prepared to simulate in many respects nasal secretions to ensure maintenance of normal ciliary action. As disclosed in Remington's Pharmaceutical Sciences (Mack Publishing, 18th Edition), which is incorporated herein by reference in its entirety, and well-known to those skilled in the art, suitable formulations are most often and preferably isotonic, slightly buffered to maintain a pH of 5.5 to 6.5, and most often and preferably include anti-microbial preservatives and appropriate drug stabilizers. Pharmaceutical formulations for intraauricular delivery include suspensions and ointments for topical application in the ear. Common solvents for such aural formulations include glycerin and water.

[0243] When used as an anti-cancer, anti-inflammatory or anti-microbial compound, for example, the compounds of Formulas I-V or compositions including Formulas I-V can be administered by either oral or non-oral pathways. When administered orally, it can be administered in capsule, tablet, granule, spray, syrup, or other such form. When administered non-orally, it can be administered as an aqueous suspension, an oily preparation or the like or as a drip, suppository, salve, ointment or the like, when administered via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, or the like.

[0244] In one embodiment, the anti-cancer, anti-inflammatory or anti-microbial can be mixed with additional substances to enhance their effectiveness. In one embodiment, the anti-microbial is combined with an additional anti-microbial. In another embodiment, the anti-microbial is combined with a drug or medicament that is helpful to a patient that is taking anti-microbials.

[0245] Methods of Administration

[0246] In an alternative embodiment, the disclosed chemical compounds and the disclosed pharmaceutical compositions are administered by a particular method as an anti-microbial. Such methods include, among others, (a) administration through oral pathways, which administration includes administration in capsule, tablet, granule, spray, syrup, or other such forms; (b) administration through non-oral pathways, which administration includes administration as an aqueous suspension, an oily preparation or the like or as a drip, suppository, salve, ointment or the like; administration via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, intradermally, or the like; as well as (c) administration topically, (d) administration rectally, or (e) administration vaginally, as deemed appropriate by those of skill in the art for bringing the compound of the present embodiment into contact with living tissue; and (f) administration via controlled released formulations, depot formulations, and infusion pump delivery. As further examples of such modes of administration

and as further disclosure of modes of administration, disclosed herein are various methods for administration of the disclosed chemical compounds and pharmaceutical compositions including modes of administration through intraocular, intranasal, and intraauricular pathways.

[0247] The pharmaceutically effective amount of the compositions that include the described compounds, including those of Formulae I-V, required as a dose will depend on the route of administration, the type of animal, including human, being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize.

[0248] In practicing the methods of the embodiment, the products or compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized *in vivo*, ordinarily in a mammal, preferably in a human, or *in vitro*. In employing them *in vivo*, the products or compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, vaginally, nasally or intraperitoneally, employing a variety of dosage forms. Such methods may also be applied to testing chemical activity *in vivo*.

[0249] As will be readily apparent to one skilled in the art, the useful *in vivo* dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular compounds employed, and the specific use for which these compounds are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, can be accomplished by one skilled in the art using routine pharmacological methods. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. Alternatively, acceptable *in vitro* studies can be used to establish useful doses and routes of administration of the compositions identified by the present methods using established pharmacological methods.

[0250] In non-human animal studies, applications of potential products are commenced at higher dosage levels, with dosage being decreased until the desired effect is no longer achieved or adverse side effects disappear. The dosage may range broadly, depending upon the desired effects and the therapeutic indication. Typically, dosages may be between about 10 microgram/kg and 100 mg/kg body weight, preferably between about 100 microgram/kg and 10 mg/kg body weight. Alternatively dosages may be based and calculated upon the surface area of the patient, as understood by those of skill in the art. Administration is preferably oral on a daily or twice daily basis.

[0251] The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. See for example, Fingl et al., in *The Pharmacological Basis of Therapeutics*, 1975, which is incorporated herein by reference in its entirety. It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to

toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

[0252] Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. A variety of techniques for formulation and administration may be found in Remington's *Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, Pa. (1990), which is incorporated herein by reference in its entirety. Suitable administration routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

[0253] For injection, the agents of the embodiment may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the embodiment into dosages suitable for systemic administration is within the scope of the embodiment. With proper choice of carrier and suitable manufacturing practice, the compositions disclosed herein, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the embodiment to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

[0254] Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external micro-environment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

[0255] Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers

comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions may be manufactured in a manner that is itself known, for example, by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

[0256] Compounds disclosed herein can be evaluated for efficacy and toxicity using known methods. For example, the toxicology of a particular compound, or of a subset of the compounds, sharing certain chemical moieties, may be established by determining *in vitro* toxicity towards a cell line, such as a mammalian, and preferably human, cell line. The results of such studies are often predictive of toxicity in animals, such as mammals, or more specifically, humans. Alternatively, the toxicity of particular compounds in an animal model, such as mice, rats, rabbits, dogs or monkeys, may be determined using known methods. The efficacy of a particular compound may be established using several art recognized methods, such as *in vitro* methods, animal models, or human clinical trials. Art-recognized *in vitro* models exist for nearly every class of condition, including the conditions abated by the compounds disclosed herein, including cancer, cardiovascular disease, and various immune dysfunction, and infectious diseases. Similarly, acceptable animal models may be used to establish efficacy of chemicals to treat such conditions. When selecting a model to determine efficacy, the skilled artisan can be guided by the state of the art to choose an appropriate model, dose, and route of administration, and regime. Of course, human clinical trials can also be used to determine the efficacy of a compound in humans.

[0257] When used as an anti-microbial, anti-cancer, or anti-inflammatory agent, the compounds disclosed herein may be administered by either oral or a non-oral pathways. When administered orally, it can be administered in capsule, tablet, granule, spray, syrup, or other such form. When administered non-orally, it can be administered as an aqueous suspension, an oily preparation or the like or as a drip, suppository, salve, ointment or the like, when administered via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, intradermally, or the like. Controlled release formulations, depot formulations, and infusion pump delivery are similarly contemplated.

[0258] The compositions disclosed herein in pharmaceutical compositions may also comprise a pharmaceutically acceptable carrier. Such compositions may be prepared for storage and for subsequent administration. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). For example, such compositions may be formulated and used as tablets, capsules or solutions for oral administration; suppositories for rectal or vaginal administration; sterile solutions or suspensions for injectable administration. Injectable can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients include, but are not limited to, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like.

In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (for example, liposomes), may be utilized.

[0259] The pharmaceutically effective amount of the composition required as a dose will depend on the route of administration, the type of animal being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize.

[0260] The products or compositions of the embodiment, as described above, may be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized *in vivo* or *in vitro*. The useful dosages and the most useful modes of administration will vary depending upon the age, weight and animal treated, the particular compounds employed, and the specific use for which these composition or compositions are employed. The magnitude of a dose in the management or treatment for a particular disorder will vary with the severity of the condition to be treated and to the route of administration, and depending on the disease conditions and their severity, the compositions may be formulated and administered either systemically or locally. A variety of techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, Pa. (1990).

[0261] To formulate the compounds of Formulas I-V as an anti-microbial, an anti-cancer, or an anti-inflammatory agent, known surface active agents, excipients, smoothing agents, suspension agents and pharmaceutically acceptable film-forming substances and coating assistants, and the like may be used. Preferably alcohols, esters, sulfated aliphatic alcohols, and the like may be used as surface active agents; sucrose, glucose, lactose, starch, crystallized cellulose, mannitol, light anhydrous silicate, magnesium aluminate, magnesium methasilicate aluminate, synthetic aluminum silicate, calcium carbonate, sodium acid carbonate, calcium hydrogen phosphate, calcium carboxymethyl cellulose, and the like may be used as excipients; magnesium stearate, talc, hardened oil and the like may be used as smoothing agents; coconut oil, olive oil, sesame oil, peanut oil, soya may be used as suspension agents or lubricants; cellulose acetate phthalate as a derivative of a carbohydrate such as cellulose or sugar, or methylacrylate-methacrylate copolymer as a derivative of polyvinyl may be used as suspension agents; and plasticizers such as ester phthalates and the like may be used as suspension agents. In addition to the foregoing preferred ingredients, sweeteners, fragrances, colorants, preservatives and the like may be added to the administered formulation of the compound produced by the method of the embodiment, particularly when the compound is to be administered orally.

[0262] The compounds and compositions may be orally or non-orally administered to a human patient in the amount of about 0.001 mg/kg/day to about 10,000 mg/kg/day of the active ingredient, and more preferably about 0.1 mg/kg/day to about 100 mg/kg/day of the active ingredient at, preferably, one time per day or, less preferably, over two to about

ten times per day. Alternatively and also preferably, the compound produced by the method of the embodiment may preferably be administered in the stated amounts continuously by, for example, an intravenous drip. Thus, for the example of a patient weighing 70 kilograms, the preferred daily dose of the active or anti-infective ingredient would be about 0.07 mg/day to about 700 gm/day, and more preferably, 7 mg/day to about 7 grams/day. Nonetheless, as will be understood by those of skill in the art, in certain situations it may be necessary to administer the anti-cancer, anti-inflammatory or the anti-infective compound of the embodiment in amounts that excess, or even far exceed, the above-stated, preferred dosage range to effectively and aggressively treat particularly advanced cancers or infections.

[0263] In the case of using the anti-microbial produced by methods of the embodiment as a biochemical test reagent, the compound produced by methods of the embodiment inhibits the progression of the disease when it is dissolved in an organic solvent or hydrous organic solvent and it is directly applied to any of various cultured cell systems. Usable organic solvents include, for example, methanol, methylsulfoxide, and the like. The formulation can, for example, be a powder, granular or other solid inhibitor, or a liquid inhibitor prepared using an organic solvent or a hydrous organic solvent. While a preferred concentration of the compound produced by the method of the embodiment for use as an anti-microbial, anticancer or anti-tumor compound is generally in the range of about 1 to about 100 $\mu\text{g/ml}$, the most appropriate use amount varies depending on the type of cultured cell system and the purpose of use, as will be appreciated by persons of ordinary skill in the art. Also, in certain applications it may be necessary or preferred to persons of ordinary skill in the art to use an amount outside the foregoing range.

[0264] In one embodiment, the method of using a compound as an anti-microbial, anti-cancer or anti-inflammatory involves administering an effective amount of -any of the compounds of Formule I-V or compositions of those compounds. In a preferred embodiment, the method involves administering the compound represented by Formula II, to a patient in need of an anti-microbial, until the need is effectively reduced or more preferably removed.

[0265] As will be understood by one of skill in the art, "need" is not an absolute term and merely implies that the patient can benefit from the treatment of the anti-microbial, the anti-cancer, or anti-inflammatory in use. By "patient" what is meant is an organism that can benefit by the use of an anti-microbial, anti-cancer or anti-inflammatory agent. For example, any organism with *B. anthracis*, *Plasmodium*, *Leishmania*, *Trypanosoma*, and the like, may benefit from the application of an anti-microbial that may in turn reduce the amount of microbes present in the patient. As another example, any organism with cancer, such as, a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma, multiple myelomas, a melanoma, and the like, may benefit from the application of an anti-cancer agent that may in turn reduce the amount of cancer present in the patient. Furthermore, any organism with an inflammatory conditions, such as, rheumatoid arthritis, asthma, multiple sclerosis, psoriasis, stroke, myocardial infarction, and the like, may benefit from the application of an anti-inflammatory that may in

turn reduce the amount of cells associated with the inflammatory response present in the patient. In one embodiment, the patient's health may not require that an anti-microbial, anti-cancer, or anti-inflammatory be administered, however, the patient may still obtain some benefit by the reduction of the level of microbes, cancer cells, or inflammatory cells present in the patient, and thus be in need. In one embodiment, the anti-microbial or anti-cancer agent is effective against one type of microbe or cancer, but not against other types; thus, allowing a high degree of selectivity in the treatment of the patient. In other embodiments, the anti-inflammatory may be effective against inflammatory conditions characterized by different cells associated with the inflammation. In choosing such an anti-microbial, anti-cancer or anti-inflammatory agent, the methods and results disclosed in the Examples may be useful. In an alternative embodiment, the anti-microbial may be effective against a broad spectrum of microbes, preferably a broad spectrum of foreign, and, more preferably, harmful bacteria, to the host organism. In embodiments, the anti-cancer and/or anti-inflammatory agent may be effective against a broad spectrum of cancers and inflammatory conditions/cells/substances. In yet another embodiment, the anti-microbial is effective against all microbes, even those native to the host. Examples of microbes that may be targets of anti-microbials, include, but are not limited to, *B. anthracis*, *Plasmodium*, *Leishmania*, *Trypanosoma*, and the like. In still further embodiments, the anti-cancer agent is effective against a broad spectrum of cancers or all cancers. Examples of cancers, against which the compounds may be effective include a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma, multiple myelomas, a melanoma, and the like. Exemplary inflammatory conditions against which the agents are effective include rheumatoid arthritis, asthma, multiple sclerosis, psoriasis, stroke, myocardial infarction, and the like.

[0266] "Therapeutically effective amount," "pharmaceutically effective amount," or similar term, means that amount of drug or pharmaceutical agent that will result in a biological or medical response of a cell, tissue, system, animal, or human that is being sought. In a preferred embodiment, the medical response is one sought by a researcher, veterinarian, medical doctor, or other clinician.

[0267] "Anti-microbial" refers to a compound that reduces the likelihood of survival of microbes, or blocks or alleviates the deleterious effects of a microbe. In one embodiment, the likelihood of survival is determined as a function of an individual microbe; thus, the anti-microbial will increase the chance that an individual microbe will die. In one embodiment, the likelihood of survival is determined as a function of a population of microbes; thus, the anti-microbial will increase the chances that there will be a decrease in the population of microbes. In one embodiment, anti-microbial means antibiotic or other similar term. Such anti-microbials are capable of blocking the harmful effects, destroying or suppressing the growth or reproduction of microorganisms, such as bacteria. For example, such antibacterials and other anti-microbials are described in Antibiotics, Chemotherapeutics and Antibacterial Agents for Disease Control (M. Grayson, editor, 1982), and E. Gale et al., The Molecular Basis of Antibiotic Action 2d edition (1981). In another embodiment, an anti-microbial will not change the likelihood of survival, but will change the chances that the

microbes will be harmful to the host in some way. For instance, if the microbe secretes a substance that is harmful to the host, the anti-microbial may act upon the microbe to stop the secretion or may counteract or block the harmful effect. In one embodiment, an anti-microbial, while, increasing the likelihood that the microbe(s) will die, is minimally harmful to the surrounding, non-microbial, cells. In an alternative embodiment, it is not important how harmful the anti-microbial is to surrounding, non-microbial, cells, as long as it reduces the likelihood of survival of the microbe.

[0268] "Anti-cancer agent" refers to a compound or composition including the compound that reduces the likelihood of survival of a cancer cell. In one embodiment, the likelihood of survival is determined as a function of an individual cancer cell; thus, the anti-cancer agent will increase the chance that an individual cancer cell will die. In one embodiment, the likelihood of survival is determined as a function of a population of cancer cells; thus, the anti-cancer agent will increase the chances that there will be a decrease in the population of cancer cells. In one embodiment, anti-cancer agent means chemotherapeutic agent or other similar term.

[0269] A "chemotherapeutic agent" is a chemical compound useful in the treatment of a neoplastic disease, such as cancer. Examples of chemotherapeutic agents include alkylating agents, such as a nitrogen mustard, an ethyleneimine and a methylmelamine, an alkyl sulfonate, a nitrosourea, and a triazene, folic acid antagonists, antimetabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, corticosteroids, a natural product such as a vinca alkaloid, an epipodophylotoxin, an antibiotic, an enzyme, a taxane, and a biological response modifier; miscellaneous agents such as a platinum coordination complex, an anthracenedione, an anthracycline, a substituted urea, a methyl hydrazine derivative, or an adrenocortical suppressant; or a hormone or an antagonist such as an adrenocorticosteroid, a progestin, an estrogen, an antiestrogen, an androgen, an antiandrogen, or a gonadotropin-releasing hormone analog. Specific examples include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytosin, Taxol, Taxotere, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincristine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Etoposides, Melphalan, and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

[0270] The anti-cancer agent may act directly upon a cancer cell to kill the cell, induce death of the cell, to prevent division of the cell, and the like. Alternatively, the anti-cancer agent may indirectly act upon the cancer cell by limiting nutrient or blood supply to the cell, for example. Such anti-cancer agents are capable of destroying or suppressing the growth or reproduction of cancer cells, such as a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma, multiple myelomas, a melanoma, and the like.

[0271] A "neoplastic disease" or a "neoplasm" refers to a cell or a population of cells, including a tumor or tissue

(including cell suspensions such as bone marrow and fluids such as blood or serum), that exhibits abnormal growth by cellular proliferation greater than normal tissue. Neoplasms can be benign or malignant.

[0272] An "inflammatory condition" includes, for example, conditions such as ischemia, septic shock, autoimmune diseases, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, multiple sclerosis, asthma, osteoarthritis, osteoporosis, fibrotic diseases, dermatosis, including psoriasis, atopic dermatitis and ultraviolet radiation (UV)-induced skin damage, psoriatic arthritis, alkylating spondylitis, tissue and organ rejection, Alzheimer's disease, stroke, atherosclerosis, stenosis, diabetes, glomerulonephritis, cancer, Hodgkins disease, cachexia, inflammation associated with infection and certain viral infections, including acquired immune deficiency syndrome (AIDS), adult respiratory distress syndrome and Ataxia Telangiectasia.

[0273] In one embodiment, a described compound, preferably a compound having the Formulas I-V, including those as described herein, is considered an effective anti-microbial, anti-cancer, or anti-inflammatory if the compound can influence 10% of the microbes, cancer cells, or inflammatory cells, for example. In a more preferred embodiment, the compound is effective if it can influence 10 to 50% of the microbes, cancer cells, or inflammatory cells. In an even more preferred embodiment, the compound is effective if it can influence 50-80% of the microbes, cancer cells, or inflammatory cells. In an even more preferred embodiment, the compound is effective if it can influence 80-95% of the microbes, cancer cells, or inflammatory cells. In an even more preferred embodiment, the compound is effective if it can influence 95-99% of the microbes, cancer cells, or inflammatory cells. "Influence" is defined by the mechanism of action for each compound. Thus, for example, if a compound prevents the reproduction of microbes, then influence is a measure of prevention of reproduction. Likewise, if a compound destroys microbes, then influence is a measure of microbe death. Also, for example, if a compound prevents the division of cancer cells, then influence is a measure of prevention of cancer cell division. Further, for example, if a compound prevents the proliferation of inflammatory cells, then influence is a measure of prevention of inflammatory cell proliferation. Not all mechanisms of action need be at the same percentage of effectiveness. In an alternative embodiment, a low percentage effectiveness may be desirable if the lower degree of effectiveness is offset by other factors, such as the specificity of the compound, for example. Thus a compound that is only 10% effective, for example, but displays little in the way of harmful side-effects to the host, or non-harmful microbes or cells, can still be considered effective.

[0274] In one embodiment, the compounds described herein are administered simply to remove microbes, cancer cells or inflammatory cells, and need not be administered to a patient. For example, in situations where microbes can present a problem, such as in food products, the compounds described herein can be administered directly to the products to reduce the risk of microbes in the products. Alternatively, the compounds can be used to reduce the level of microbes present in the surrounding environment, such working surfaces. As another example, the compounds can be administered ex vivo to a cell sample, such as a bone marrow or stem

cell transplant to ensure that only non-cancerous cells are introduced into the recipient. After the compounds are administered they may optionally be removed. This may be particularly desirable in situations where work surfaces or food products may come into contact with other surfaces or organisms that could risk being harmed by the compounds. In an alternative embodiment, the compounds may be left in the food products or on the work surfaces to allow for a more protection. Whether or not this is an option will depend upon the relative needs of the situation and the risks associated with the compound, which in part can be determined as described in the Examples below.

[0275] The following non-limiting examples are meant to describe the preferred embodiments of the methods. Variations in the details of the particular methods employed and in the precise chemical compositions obtained will undoubtedly be appreciated by those of skill in the art.

EXAMPLES

Example 1

Fermentation of Compound of Formulae II-16, II-20, and II-24C

[0276] Strain CNB476 was grown in a 500-ml flask containing 100 ml of vegetative medium consisting of the following per liter of deionized water: glucose, 4 g; Bacto tryptone, 3 g; Bacto casitone, 5 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28 degree C. for 3 days on a rotary shaker operating at 250 rpm. Four ml each of the first seed culture was inoculated into three 500-ml flasks containing of 100 ml of the vegetative medium. The second seed cultures were incubated at 28 degree C. and 250 rpm on a rotary shaker for 2 days. Four ml each of the second seed culture was inoculated into thirty-five 500-ml flasks containing of 100 ml of the vegetative medium. The third seed cultures were incubated at 28 degree and 250 rpm on a rotary shaker for 2 days. Four ml each of the third seed culture was inoculated into four hundred 500-ml flasks containing 100 ml of the production medium consisting of the following per liter of deionized water: starch, 10 g; yeast extract, 4 g; Hy-Soy, 4 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The production cultures were incubated at 28 degree C. and 250 rpm on rotary shakers for 1 day. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were added to the production cultures. The production cultures were further incubated at 28 degree C. and 250 rpm on rotary shakers for 5 days. The culture broth was filtered through cheese cloth to recover the Amberlite XAD-7 resin. The resin was extracted with 2 times 6 liters ethyl acetate followed by 1 time 1.5 liters ethyl acetate. The combined extracts were dried in vacuo. The dried extract, containing 3.8 grams the compound of Formula II-16 and lesser quantities of compounds of formulae II-20 and II-24C, was then processed for the recovery of the compounds of Formula II-16, II-20 and II-24C.

Example 2

Purification of Compound of Formulae II-16, II-20 and II-24C

[0277] The pure compounds of Formulae II-16, II-20 and II-24C were obtained by flash chromatography followed by

HPLC. Eight grams crude extract containing 3.8 grams of the compound of Formula II-16 and lesser quantities of II-20 and II-24C was processed by flash chromatography using Biotage Flash40i system and Flash 40M cartridge (KP-Sil Silica, 32-63 μ m, 90 grams). The flash chromatography was developed by the following step gradient:

- [0278] 1. Hexane (1 L)
- [0279] 2. 10% Ethyl acetate in hexane (1 L)
- [0280] 3. 20% Ethyl acetate in hexane, first elution (1 L)
- [0281] 4. 20% Ethyl acetate in hexane, second elution (1 L)
- [0282] 5. 20% Ethyl acetate in hexane, third elution (1 L)
- [0283] 6. 25% Ethyl acetate in hexane (1 L)
- [0284] 7. 50% Ethyl acetate in hexane (1 L)
- [0285] 8. Ethyl acetate (1 L)

[0286] Fractions containing the compound of Formula II-16 in greater or equal to 70% UV purity by HPLC were pooled and subject to HPLC purification, as described below, to obtain II-16, along with II-20 and II-24C, each as pure compounds

Column	Phenomenex Luna 10u Silica
Dimensions	25 cm x 21.2 mm ID
Flow rate	25 ml/min
Detection	ELSD
Solvent	Gradient of 24% EtOAc/hexane for 19 min, 24% EtOAc/hexane to 100% EtOAc in 1 min, then 100% EtOAc for 4 min

[0287] The fraction enriched in compound of Formula II-16 (described above; ~70% pure with respect to II-16) was dissolved in acetone (60 mg/ml). Aliquots (950 μ l) of this solution were injected onto a normal-phase HPLC column using the conditions described above. The compound of Formula II-16 eluted at about 14 minutes, and minor compounds II-24C and II-20 eluted at 11 and 23 minutes, respectively. Fractions containing II-16, II-24C, and II-20 were pooled based on composition of compound present. Fractions containing the desired compounds were concentrated under reduced pressure to yield pure compound of Formula II-16, as well as separate fractions containing II-24C and II-20, which were further purified as described below.

[0288] Sample containing II-24C (70 mg) was dissolved in acetonitrile at a concentration of 10 mg/ml, and 500 μ l was loaded on an HPLC column of dimensions 21 mm i.d. by 15 cm length containing Eclipse XDB-C18 support. The solvent gradient increased linearly from 15% acetonitrile/85% water to 100% acetonitrile over 23 minutes at a flow rate of 14.5 ml/min. The solvent composition was held at 100% acetonitrile for 3 minutes before returning to the starting solvent mixture. Compound II-24C eluted at 19 minutes as a pure compound under these conditions.

[0289] To obtain pure compound II-20, the enriched samples generated from the preparative HPLC method

described above were triturated with EtOAc to remove minor lipophilic impurities. The resulting sample contained compound II-20 in >95% purity.

[0290] Compound of Formula II-16: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 314 (M+H), 336 (M+Na).

[0291] Compound of Formula II-20: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 266 (M+H). **FIG. 7** depicts the 1H NMR spectrum of a compound having the structure of Formula II-20.

[0292] Compound of Formula II-24C: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 328 (M+H), 350 (M+Na). **FIG. 8** depicts the 1H NMR spectrum of a compound having the structure of Formula II-24C.

Example 3

Fermentation of Compounds of Formulae II-17 and II-18

[0293] Strain CNB476 was grown in a 500-ml flask containing 100 ml of the first vegetative medium consisting of the following per liter of deionized water: glucose, 4 g; Bacto tryptone, 3 g; Bacto casitone, 5 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28 degree C. for 3 days on a rotary shaker operating at 250 rpm. Five ml of the first seed culture was inoculated into a 500-ml flask containing 100 ml of the second vegetative medium consisting of the following per liter of deionized water: starch, 10 g; yeast extract, 4 g; peptone, 2 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and sodium bromide, 30 g. The second seed cultures were incubated at 28° C. for 7 days on a rotary shaker operating at 250 rpm. Approximately 2 to 3 gram of sterile Amberlite XAD-7 resin were added to the second seed culture. The second seed culture was further incubated at 28° C. for 2 days on a rotary shaker operating at 250 rpm. Five ml of the second seed culture was inoculated into a 500-ml flask containing 100 ml of the second vegetative medium. The third seed culture was incubated at 28° C. for 1 day on a rotary shaker operating at 250 rpm. Approximately 2 to 3 gram of sterile Amberlite XAD-7 resin were added to the third seed culture. The third seed culture was further incubated at 28° C. for 2 days on a rotary shaker operating at 250 rpm. Five ml of the third culture was inoculated into a 500-ml flask containing 100 ml of the second vegetative medium. The fourth seed culture was incubated at 28° C. for 1 day on a rotary shaker operating at 250 rpm. Approximately 2 to 3 gram of sterile Amberlite XAD-7 resin were added to the fourth seed culture. The fourth seed culture was further incubated at 28° C. for 1 day on a rotary shaker operating at 250 rpm. Five ml each of the fourth seed culture was inoculated into ten 500-ml flasks containing 100 ml of the second vegetative medium. The fifth seed cultures were incubated at 28° C. for 1 day on a rotary shaker operating at 250 rpm. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were added to the fifth seed cultures. The fifth seed cultures were further incubated at 28° C. for 3 days on a rotary shaker operating at 250 rpm. Four ml each of the fifth seed culture was inoculated into one hundred and fifty 500-ml flasks containing 100 ml of the production medium having the same composition as the second vegetative medium. Approxi-

mately 2 to 3 grams of sterile Amberlite XAD-7 resin were also added to the production culture. The production cultures were incubated at 28° C. for 6 day on a rotary shaker operating at 250 rpm. The culture broth was filtered through cheese cloth to recover the Amberlite XAD-7 resin. The resin was extracted with 2 times 3 liters ethyl acetate followed by 1 time 1 liter ethyl acetate. The combined extracts were dried in vacuo. The dried extract, containing 0.42 g of the compound Formula II-17 and 0.16 gram the compound of Formula II-18, was then processed for the recovery of the compounds.

Example 4

Purification of Compounds of Formula II-17 and II-18

[0294] The pure compounds of Formula II-17 and II-18 were obtained by reversed-phase HPLC as described below:

Column	ACE 5 C18-HL
Dimensions	15 cm x 21 mm ID
Flow rate	14.5 ml/min
Detection	214 nm
Solvent	Gradient of 35% Acetonitrile/65% H_2O to 90% Acetonitrile/10% H_2O over 15 min

[0295] Crude extract (100 mg) was dissolved in 15 ml of acetonitrile. Aliquots (900 μ l) of this solution were injected onto a reversed-phase HPLC column using the conditions described above. Compounds of Formulae II-17 and II-18 eluted at 7.5 and 9 minutes, respectively. Fractions containing the pure compounds were first concentrated using nitrogen to remove organic solvent. The remaining solution was then frozen and lyophilized to dryness.

[0296] Compound of Formula II-17: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm. High Res. Mass (APCI): m/z 280.156 (M+H), Δ_{calc} =2.2 ppm, $C_{15}H_{22}NO_4$. **FIG. 49** depicts the 1H NMR spectrum of a compound having the structure of Formula II-17.

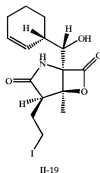
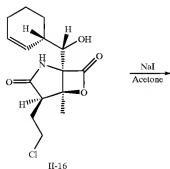
[0297] Compound of Formula II-18: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm. High Res. Mass (APCI): m/z 358.065 (M+H), Δ_{calc} =-1.9 ppm, $C_{15}H_{21}NO_4Br$. **FIG. 50** depicts the 1H NMR spectrum of a compound having the structure of Formula II-18.

Example 5

Preparation of Compound of Formula II-19 from II-16

[0298] A sample of compound of Formula II-16 (250 mg) was added to an acetone solution of sodium iodide (1.5 g in 10 ml) and the resulting mixture stirred for 6 days. The solution was then filtered through a 0.45 micron syringe filter and injected directly on a normal phase silica HPLC column (Phenomenex Luna 10 μ Silica, 25 cmx21.2 mm) in 0.95 ml aliquots. The HPLC conditions for the separation of compound Formula II-19 from unreacted II-16 employed an isocratic HPLC method consisting of 24% ethyl acetate and 76% hexane, in which the majority of compound II-19 eluted 2.5 minutes before compound II-16. Equivalent frac-

tions from each of 10 injections were pooled to yield 35 mg compound II-19. Compound II-19: UV (Acetonitrile/H₂O) 225 (sh), 255 (sh) nm; ESMS, m/z 406.0 (M+H); ¹H NMR in DMSO-d₆ (see FIG. 9).

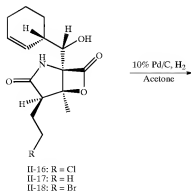


Example 6

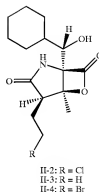
Synthesis of the Compounds of Formulae II-2, II-3, and II-4

[0299] Compounds of Formulae II-2, II-3 and II-4 can be synthesized from compounds of Formulae II-16, II-17 and II-18, respectively, by catalytic hydrogenation.

[0300] Exemplary Depiction of Synthesis



-continued



Example 6A

Catalytic Hydrogenation of Compound of Formula II-16

[0301] Compound of Formula II-16 (10 mg) was dissolved in acetone (5 mL) in a scintillation vial (20 mL) to which was added the 10% (w/w) Pd/C (1-2mg) and a magnetic stirrer bar. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 3 cc silica column and washed with acetone. The filtrate was filtered again through 0.2 μ m Gelman Acrodisc to remove any traces of catalyst. The solvent was evaporated off from filtrate under reduced pressure to yield the compound of Formula II-2 as a pure white powder: UV (acetonitrile/H₂O): λ_{max} 225 (sh) nm. FIG. 10 depicts the NMR spectrum of the compound of Formula II-2 in DMSO-d₆. FIG. 11 depicts the low resolution mass spectrum of the compound of Formula II-2: m/z 316 (M+H), 338 (M+Na).

Example 6B

Catalytic Hydrogenation of Compound of Formula II-17

[0302] Compound of Formula II-17 (5 mg) was dissolved in acetone (3 mL) in a scintillation vial (20 mL) to which was added the 10% (w/w) Pd/C (about 1mg) and a magnetic stirrer bar. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodisc to remove the catalyst. The solvent was evaporated off from filtrate to yield the compound of Formula II-3 as a white powder which was purified by normal phase HPLC using the following conditions:

Column:	Phenomenex Luna 10u Silica
Dimensions:	25 cm x 21.2 mm ID
Flow rate:	14.5 mL/min
Detection:	ELSD
Solvent:	5% to 60% EtOAc/Hex for 19 min, 60 to 100% EtOAc in 1 min, then 4 min at 100% EtOAc

[0303] Compound of Formula II-3 eluted at 22.5 min as a pure compound: UV (acetonitrile/H₂O): λ_{\max} 225 (sh) nm. **FIG. 12** depicts the NMR spectrum of the compound of Formula II-3 in DMSO-d₆. **FIG. 13** depicts the low resolution mass spectrum of the compound of Formula II-3: m/z 282 (M+H), 304 (M+Na).

Example 6C

Catalytic Hydrogenation of Compound of Formula II-18

[0304] 3.2 mg of compound of Formula II-18 was dissolved in acetone (3 mL) in a scintillation vial (20 mL) to which was added the 10% (w/w) Pd/C (about 1 mg) and a magnetic stirrer bar. The reaction mixture was stirred in hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodisc to remove the catalyst. The solvent was evaporated off from filtrate to yield the compound of Formula II-4 as a white powder which was further purified by normal phase HPLC using the following conditions:

Column:	Phenomenex Luna 10u Silica
Dimensions:	25 cm \times 21.2 mm ID
Flow rate:	14.5 mL/min
Detection:	ELSD
Solvent:	5% to 80% EtOAc/Hex for 19 min, 80 to 100% EtOAc in 1 min, then 4 min at 100% EtOAc

[0305] Compound of Formula II-4 eluted at 16.5 min as a pure compound: UV (acetonitrile/H₂O): λ_{\max} 225 (sh) nm. **FIG. 14** depicts the NMR spectrum of the compound of Formula II-4 in DMSO-d₆. **FIG. 15** depicts the low resolution mass of the compound of Formula II-4: m/z 360 (M+H), 382 (M+Na).

Example 7

Synthesis of the Compounds of Formulae II-5A and II-5B

[0306] Compounds of Formula II-5A and Formula II-5B can be synthesized from compound of Formula II-16 by epoxidation with mCPBA.

[0307] Compound of Formula II-16 (101 mg, 0.32 mmole) was dissolved in methylenechloride (30 mL) in a 100 mL of round bottom flask to which was added 79 mg (0.46 mmole) of meta-chloroperoxybenzoic acid (mCPBA) and a magnetic stir bar. The reaction mixture was stirred at room temperature for about 18 hours. The reaction mixture was poured onto a 20 cc silica flash column and eluted with 120 mL of CH₂Cl₂, 75 mL of 1:1 ethyl acetate/hexane and finally with 40 mL of 100% ethyl acetate. The 1:1 ethyl acetate/hexane fractions yield a mixture of diastereomers of epoxyderivatives, Formula II-5A and II-5B, which were separated by normal phase HPLC using the following conditions:

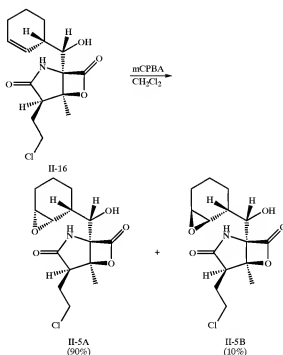
Column	Phenomenex Luna 10u Silica
Dimensions	25 cm \times 21.2 mm ID
Flow rate	14.5 mL/min
Detection	ELSD

-continued

Solvent	25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc in 1 min, then 5 min at 100% EtOAc
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[0308] Compound Formula II-5A (major product) and II-5B (minor product) eluted at 21.5 and 19 min, respectively, as pure compounds. Compound II-5B was further chromatographed on a 3cc silica flash column to remove traces of chlorobenzoic acid reagent.

Chemical Structures:



[0309] Structural Characterization

[0310] Formula II-5A: UV (Acetonitrile/H₂O) λ_{\max} 225 (sh) nm. Low Res. Mass: m/z 330 (M+H), 352 (M+Na). **FIGS. 16-17**, respectively depict the 1H NMR spectrum of Formula II-5A and the mass spectrum of Formula II-5A.

[0311] Formula II-5B: UV (Acetonitrile/H₂O) λ_{\max} 225 (sh) nm. Low Res. Mass: m/z 330 (M+H), 352 (M+Na). **FIGS. 18-19**, respectively depict the 1H NMR spectrum of II-5B and the mass spectrum of II-5B.

Example 8

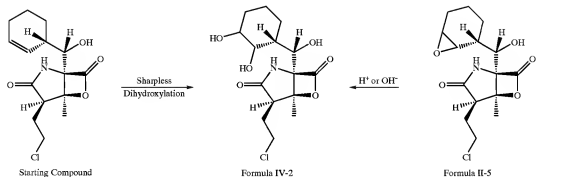
Synthesis of the Compounds of Formulae IV-1 IV-2, IV-3 and IV-4 Synthesis of Diol Derivatives (Formula IV-2)

[0312] Diols may be synthesized by Sharpless dihydroxylation using AD mix- α and β : AD mix- α is a premix of four reagents, K₂OsO₈(OH)₄, K₂CO₃, K₂Fe(CN)₆, (DHQD)-PHAL [1,4-bis(9-*O*-dihydroquinone)phthalazine] and AD

mix- β is a premix of $K_2OsO_4(OH)_4$; K_2CO_3 ; $K_3Fe(CN)_6$; (DHQD) $_2$ -PHAL. [1,4-bis(9-O-dihydroquinidine)phthalazine] which are commercially available from Aldrich. Diol can also be synthesized by acid or base hydrolysis of epoxy compounds (Formula II-5A and II-5B) which may be different to that of products obtained in Sharpless dihydroxylation in their stereochemistry at carbons bearing hydroxyl groups

[0313] Sharpless Dihydroxylation of Compounds II-16 II-17 and II-18

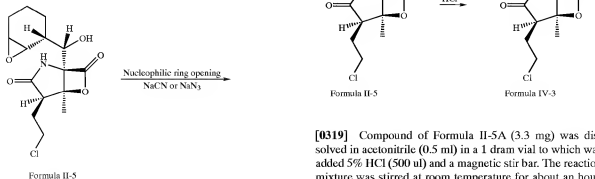
[0314] Any of the compounds of Formulae II-16, II-17 and II-18 may be used as the starting compound. In the example below, compound of Formula II-16 is used. The starting compound is dissolved in t-butanol/water in a round bottom flask to which is added AD mix- α or β and a magnetic stir bar. The reaction is monitored by silica TLC as well as mass spectrometer. The pure diols are obtained by usual workup and purification by flash chromatography or HPLC. The structures are confirmed by NMR spectroscopy and mass spectrometry. In this method both hydroxyl groups are on same side.



[0315] Nucleophilic Ring Opening of Epoxy Compounds (II-5):

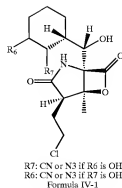
[0316] The epoxy ring is opened with various nucleophiles like $NaCN$, NaN_3 , $NaOAc$, HBr , HCl , etc. to create various substituents on the cyclohexane ring, including a hydroxyl substituent.

[0317] Examples:

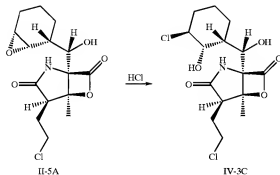


[0319] Compound of Formula II-5A (3.3 mg) was dissolved in acetonitrile (0.5 ml) in a 1 dram vial to which was added 5% HCl (500 μ l) and a magnetic stir bar. The reaction mixture was stirred at room temperature for about an hour. The reaction was monitored by mass spectrometry. The

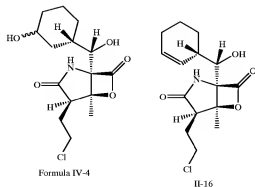
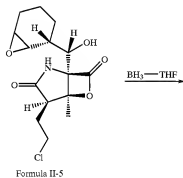
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reaction mixture was directly injected on normal phase HPLC to obtain compound of Formula IV-3C as a pure compound without any work up. The HPLC conditions used for the purification were as follows: Phenomenex Luna 10 u Silica column (25 cm×21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc in 1 min, then 5 min at 100% EtOAc at a flow rate of 14.5 ml/min. An ELSD was used to monitor the purification process. Compound of Formula IV-3C eluted at about 18 min (2.2 mg). Compound of Formula IV-3C: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm; ESMS, *m/z* 366 (M+H)⁺, 388 (M+Na)⁺; ¹H NMR in DMSO-*d*₆ (FIG. 20) The stereochemistry of the compound of Formula IV-3C was determined based on coupling constants observed in the cyclohexane ring in 1:1 C₆D₆/DMSO-*d*₆ (FIG. 21)



[0320] Reductive ring opening of epoxides (II-5): The compound of Formula is treated with metalhydrides like BH₃-THF complex to make compound of Formula IV-4.

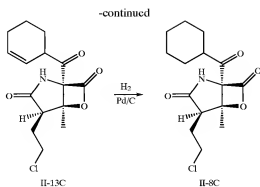


Example 9

Synthesis of the Compounds of Formulae II-13C and II-8C

[0321] Compound of Formula II-16 (30 mg) was dissolved in CH₂Cl₂ (6 ml) in a scintillation vial (20 ml) to which Dess-Martin Periodinane (122 mg) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 2 hours. The progress of the reaction was monitored by TLC (Hex:EtOAc, 6:4) and analytical HPLC. From the reaction mixture, the solvent volume was reduced to one third, absorbed on silica gel, poured on top of a 20 cc silica flash column and eluted in 20 ml fractions using a gradient of Hexane/EtOAc from 10 to 100%. The fraction eluted with 30% EtOAc in Hexane contained a mixture of rotamers of Formula II-13C in a ratio of 1.5:8.5. The mixture was further purified by normal phase HPLC using the Phenomenex Luna 10 u Silica column (25 cm×21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc over 1 min, holding at 100% EtOAc for 5 min, at a flow rate of 14.5 ml/min. An ELSD was used to monitor the purification process. Compound of Formula II-13C eluted at 13.0 and 13.2 mins as a mixture of rotamers with in a ratio of 1.5:8.5 (7 mg). Formula II-13C: UV (Acetonitrile/H₂O) λ_{max} 226 (sh) & 300 (sh) nm; ESMS, *m/z* 312 (M+H)⁺, 334 (M+Na)⁺; ¹H NMR in DMSO-*d*₆ (see FIG. 22).

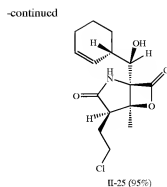
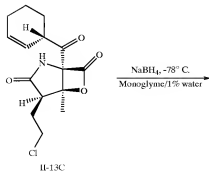
[0322] The rotamer mixture of Formula II-13C (4 mg) was dissolved in acetone (1 ml) in a scintillation vial (20 ml) to which a catalytic amount (0.5 mg) of 10% (w/w) Pd/C and a magnetic stir bar were added. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodisc to remove the catalyst. The solvent was evaporated from the filtrate to yield compound of Formula II-8C as a colorless gum which was further purified by normal phase HPLC using a Phenomenex Luna 10 u Silica column (25 cm×21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc over 1 min, holding at 100% EtOAc for 5 min, at a flow rate of 14.5 ml/min. An ELSD was used to monitor the purification process. Compound of Formula II-8C (1 mg) eluted at 13.5 min as a pure compound. Formula II-8C: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm; ESMS, *m/z* 314 (M+H)⁺, 336 (M+Na)⁺; ¹H NMR in DMSO-*d*₆ (see FIG. 23).



Example 10

Synthesis of the Compound of Formulae II-25 from II-13C

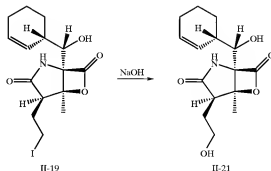
[0323] The rotamer mixture of Formula II-13C (5 mg) was dissolved in dimethoxy ethane (monoglyme; 1.5 ml) in a scintillation vial (20 ml) to which water (15 μ l (1% of the final solution concentration)) and a magnetic stir bar were added. The above solution was cooled to -78° C. on a dry ice-acetone bath, and a sodium borohydride solution (3.7 mg of NaBH_4 in 0.5 ml of monoglyme (created to allow for slow addition)) was added drop-wise. The reaction mixture was stirred at -78° C. for about 14 minutes. The reaction mixture was acidified using 2 ml of 4% HCl solution in water and extracted with CH_2Cl_2 . The organic layer was evaporated to yield mixture of compound of formulae II-25 and II-16 in a 9.5:0.5 ratio as a white solid, which was further purified by normal phase HPLC using a Phenomenex Luna 10 u Silica column (25 cm \times 21.2 mm ID). The mobile phase was 24% EtOAc/76% Hexane, which was held isocratic for 19 min, followed by a linear gradient of 24% to 100% EtOAc over 1 min, and held at 100% EtOAc for 3 min; the flow rate was 25 ml/min. An ELSD was used to monitor the purification process. Compound of formula II-25 (1.5 mg) eluted at 11.64 min as a pure compound. Compound of Formula II-25: UV (Acetonitrile/ H_2O) λ_{max} 225 (sh) nm; ESMS, m/z 314 (M+H) $^{+}$, 336 (M+Na) $^{+}$; ^1H NMR in $\text{DMSO}-d_6$ (see FIG. 24).



Example 11

[0324] Synthesis of the Compound of Formulae II-21 from II-19

[0325] Acetone (7.5 ml) was vigorously mixed with 5 N NaOH (3 ml) and the resulting mixture evaporated to a minimum volume in vacuo. A sample of 100 l of this solution was mixed with compound of Formula II-19 (6.2 mg) in acetone (1 ml) and the resulting biphasic mixture vortexed for 2 minutes. The reaction solution was immediately subjected to preparative C18 HPLC. Conditions for the purification involved a linear gradient if 10% acetonitrile/90% water to 90% acetonitrile/10% water over 17 minutes using an Ace 5 μ C18 HPLC column of dimensions 22 mm id by 150 mm length. Compound of Formula II-21 eluted at 9.1 minutes under these conditions to yield 0.55 mg compound. Compound of Formula II-21: UV (Acetonitrile/ H_2O) 225 (sh), ESMS, m/z 296.1 (M+H) $^{+}$; ^1H NMR in $\text{DMSO}-d_6$ (see FIG. 25).

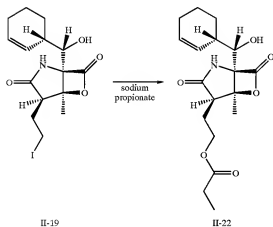


Example 12

Synthesis of the Compound of Formulae II-22 from II-19

[0326] A sample of 60 mg sodium propionate was added to a solution of compound of Formula II-19 (5.3 mg) in

DMSO (1 ml) and the mixture sonicated for 5 minutes, though the sodium propionate did not completely dissolve. After 45 minutes, the solution was filtered through a 0.45 [syringe filter and purified directly using HPLC. Conditions for the purification involved a linear gradient of 10% acetonitrile/90% water to 90% acetonitrile/10% water over 17 minutes using an Ace 5 μ C18 HPLC column of dimensions 22 mm id by 150 mm length. Under these conditions, compound of Formula II-22 eluted at 12.3 minutes to yield 0.7 mg compound (15% isolated yield). UV (Acetonitrile/H₂O) 225 (sh), ESMS, *m/z* 352.2 (M+H); ¹H NMR in DMSO-*d*₆ (see FIG. 26).

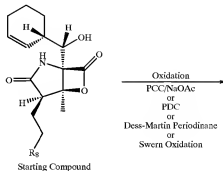


Example 13

Oxidation of Secondary Hydroxyl Group in
Compounds of Formulae II-16, II-17 and II-18 and
Reaction with Hydroxy or Methoxy Amines

[0327] Any of the compounds of Formulae II-16, II-17 and II-18 may be used as the starting compound. The secondary hydroxyl group in the starting compound is oxidized using either of the following reagents: pyridinium dichromate (PDC), pyridinium chlorochromate (PCC), Dess-Martin periodinane or oxalyl chloride (Swern oxidation) (Ref: Organic Syntheses, collective volumes I-VIII). Preferably, Dess-Martin periodinane may be used as a reagent for this reaction. (Ref: Fenteany G. et al. Science, 1995, 268, 726-73). The resulting keto compound is treated with hydroxylamine or methoxy amine to generate oximes.

[0328]



Examples

Oxidation
PCC/NaOAc
or
PDC
or
Dess-Martin Periodinane
or
Swern Oxidation

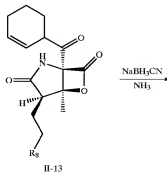
Example 14

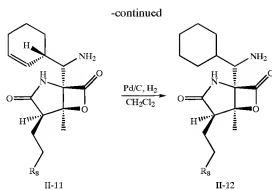
Reductive Amination of Keto-Derivative

[0329] The keto derivatives, for example Formula II-8 and II-13, are treated with sodium cyanoborohydride (NaBH₃CN) in the presence of various bases to yield amine derivatives of the starting compounds which are subsequently hydrogenated with 10% Pd/C, H₂ to reduce the double bond in the cyclohexene ring.

Example

[0330]





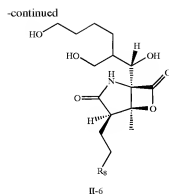
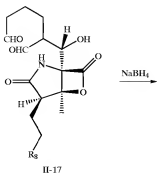
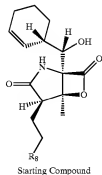
Example 15

Cyclohexene Ring Opening

[0331] Any compound of Formulae II-16, II-17 and II-18 may be used as a starting compound. The starting compound is treated with OsO_4 and NaIO_4 in $\text{THF-H}_2\text{O}$ solution to yield dial derivatives which are reduced to alcohol with NaBH_4 in the same pot.

Example

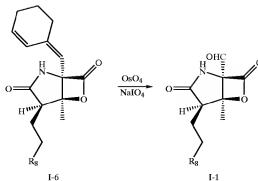
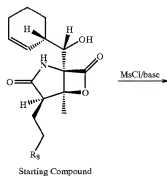
[0332]



Example 16

Dehydration of Alcohol Followed by Aldehyde Formation at Lactone-Lactam Ring Junction

[0333] A starting compound of any of Formulae II-16, II-17 or II-18 is treated with mesylchloride in the presence of base to yield a dehydrated derivative. The resulting dehydrated compound is treated with OsO_4 and NaIO_4 in $\text{THF-H}_2\text{O}$ to yield an aldehyde group at the lactone-lactam ring junction.



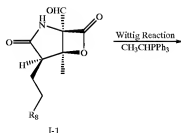
Example 17

Various Reactions on Aldehyde Derivatives I-1

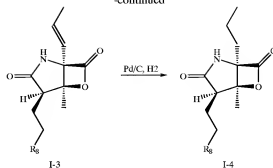
[0334] Wittig reactions are performed on the aldehyde group using various phosphorus ylides [e.g., (triphenylphosphoranylidene)ethane] to yield an olefin. The double bond in the side chain is reduced by catalytic hydrogenation.

Example

[0335]



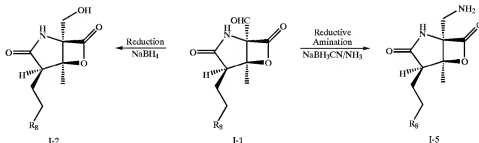
-continued



[0336] Reductive amination is performed on the aldehyde group using various bases (e.g. NH_3) and sodium cyanoborohydride to yield amine derivatives. Alternatively, the aldehyde is reduced with NaBH_4 to form alcohols in the side chain.

Example

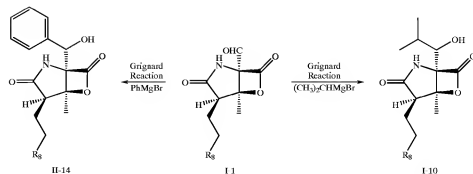
[0337]



[0338] Organometallic addition reactions to the aldehyde carbonyl, such as Grignard reactions, may be performed using various alkyl magnesium bromide or chloride reagents (e.g. isopropylmagnesium bromide, phenylmagnesium bromide) to yield various substituted secondary alcohols.

Example

[0339]



Example 18

In Vitro Biology

[0340] Initial studies of a compound of Formula II-16, which is also referred to as Salinosporamide A, employed the National Cancer Institute (NCI) screening panel, which consists of 60 human tumor cell lines that represent leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney. A detailed description of the screening procedure can be found at hypertext transfer protocol (<http://dtp.nci.nih.gov/branches/btb/iv-clsp.html>.)

[0341] In brief, each of the 60 human tumor cell lines were grown in RPMI 1640 medium, supplemented with 5% fetal bovine serum and 2 mM L-glutamine. Cells were plated at their appropriate density in 96-well microtiter plates and incubated at 37° C., 5% CO₂, 95% air and 100% relative humidity. After 24 hours, 100 pL of various 10-fold serial dilutions of Salinosporamide A were added to the appropriate wells containing 100 μ L of cells, resulting in a final Salinosporamide A concentration ranging from 10 nM to 100 μ M. Cells were incubated for an additional 48 hours and a sulforhodamine B protein assay was used to estimate cell viability or growth.

[0342] Three dose response parameters were calculated as follows:

[0343] GI₅₀ indicates the concentration that inhibits growth by 50%.

[0344] TGI indicates the concentration that completely inhibits growth.

[0345] LC₅₀ indicates the concentration that is lethal to 50% of the cells.

[0346] An example of a study evaluating Salinosporamide A in the NCI screen is shown in Table I below.

[0347] Data indicate that the mean GI₅₀ value of Salinosporamide A was less than 10 nM. The wide range (>1000-fold difference) observed in both the mean TGI and mean LC₅₀ values for the most sensitive and the most resistant tumor cell lines illustrates that Salinosporamide A displays good selectivity and does not appear to be a general toxin. Furthermore, the mean TGI data suggest that Salinosporamide A shows preferred specificity towards melanoma and breast cancer cell lines. The assay was repeated and showed similar results.

[0348] The results of the NCI tumor screen show that Salinosporamide A: (1) is a potent compound with a mean GI₅₀ value of <10 nM, and (2) displays good tumor selectivity of more than 1000-fold difference in both the mean TGI and mean LC₅₀ values between the most sensitive and resistant tumor cell lines.

Example 19

Growth Inhibition of Tumor Cell Lines

[0349] B16-F10 (ATCC; CRL-6475), DU 145 (ATCC; HTB-81), HEK293 (ATCC; CRL-1573), HT-29 (ATCC; HTB-38), LoVo (ATCC; CCL-229), MDA-MB-231 (ATCC; HTB-26), MIA PaCa-2 (ATCC; CRL-1420), NCI-H292 (ATCC; CRL-1848), OVCAR-3 (ATCC; HTB-161), PANC-1 (ATCC; CRL-1469), PC-3 (ATCC; CRL-1435), RPMI 8226 (ATCC; CCL-155) and U266 (ATCC; TIB-196) were maintained in appropriate culture media. The cells were cultured in an incubator at 37 °C. in 5% CO₂ and 95% humidified air.

[0350] For cell growth inhibition assays, B16-F10, DU 145, HEK293, HT-29, LoVo, MDA-MB-231, MIA PaCa-2, NCI-H292, OVCAR-3, PANC-1, PC-3, RPMI 8226 and U266 cells were seeded at 1.25×10^3 , 5×10^3 , 1.5×10^4 , 5×10^3 , 5×10^3 , 1×10^4 , 2×10^3 , 4×10^3 , 1×10^4 , 7.5×10^3 , 5×10^3 , 2×10^4 , 2.5×10^3 cells/well respectively in 90 μ l complete media into Corning 3904 black-walled, clear-bottom tissue culture plates. 20 mM stock solutions of Formula II-16 were prepared in 100% DMSO, aliquoted and stored at -80 °C. Formula II-16 was serially diluted and added in triplicate to the test wells resulting in final concentrations ranging from 0.2 μ M to 0.2 μ M. The plates were returned to the incubator for 48 hours. The final concentration of DMSO was 0.25% in all samples.

[0351] Following 48 hours of drug exposure, 10 μ l of 0.2 mg/ml resazurin (obtained from Sigma-Aldrich Chemical Co.) in Mg²⁺, Ca²⁺ free phosphate buffered saline was added to each well and the plates were returned to the incubator for 3-6 hours. Since living cells metabolize Resazurin, the fluorescence of the reduction product of Resazurin was measured using a Fusion microplate fluorometer (Packard Bioscience) with λ_{ex} =535 nm and λ_{em} =590 nm filters. Resazurin dye in medium without cells was used to determine the background, which was subtracted from the data for all experimental wells. The data were normalized to the average fluorescence of the cells treated with media +0.25% DMSO (100% cell growth) and EC₅₀ values (the drug concentration at which 50% of the maximal observed growth inhibition is established) were determined using a standard sigmoidal dose response curve fitting algorithm (generated by XLfit 3.0, ID Business Solutions Ltd or Prism 3.0, GraphPad Software Inc).

[0352] The data in Table 2 summarize the growth inhibitory effects of Formula II-16 against 13 diverse human and mouse tumor cell lines.

TABLE 2

Mean EC ₅₀ values of Formula II-16 against various tumor cell lines				
Cell line	Source	EC ₅₀ (nM), mean \pm SD*	n	
B16-F10	Mouse, melanoma	47 \pm 20	12	
DU 145	Human, prostate carcinoma	37 \pm 10	3	
HEK293	Human, embryonic kidney	47	2	
HT-29	Human, colorectal adenocarcinoma	40 \pm 26	5	
LoVo	Human, colorectal adenocarcinoma	70 \pm 8	3	
MDA-MB-231	Human, breast adenocarcinoma	87 \pm 40	12	

TABLE 2-continued

Mean EC ₅₀ values of Formula II-16 against various tumor cell lines				
Cell line	Source	EC ₅₀ (nM), mean \pm SD*	n	
MIA PaCa-2	Human, pancreatic carcinoma	46	2	
NCI-H292	Human, non small cell lung carcinoma	66 \pm 29	12	
OVCAR-3	Human, ovarian adenocarcinoma	49 \pm 31	6	
PANC-1	Human, pancreatic carcinoma	60	2	
PC-3	Human, prostate adenocarcinoma	64 \pm 26	19	
RPMI 8226	Human, multiple myeloma	8.6 \pm 1.9	26	
U266	Human, multiple myeloma	4.7 \pm 0.7	6	

*Where n (number of independent experiments) = 2, the mean value is presented

[0353] The EC₅₀ values indicate that Formula II-16 was cytotoxic against B16-F10, DU 145, HEK293, HT-29, LoVo, MDA-MB-231, MIA PaCa-2, NCI-H292, OVCAR-3, PANC-1, PC-3, RPMI 8226 and U266 cells.

Example 20

In vitro Inhibition of Proteasome Activity by Formulae II-2, II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-16, II-17, II-18, II-19, II-20, II-21, II-22, II-24C, II-25 and IV-3C

[0354] All the compounds were prepared as 20 mM stock solution in DMSO and stored in small aliquots at -80 °C. Purified rabbit muscle 20S proteasome was obtained from CalBiochem. To enhance the chymotrypsin-like activity of the proteasome, the assay buffer (20 mM HEPES, pH7.3, 0.5 mM EDTA, and 0.05% Triton X100) was supplemented with SDS resulting in a final SDS concentration of 0.035%. The substrate used was suc-LIVY-AMC, a fluorogenic peptide substrate specifically cleaved by the chymotrypsin-like activity of the proteasome. Assays were performed at a proteasome concentration of 1 μ g/ml in a final volume of 200 μ l in 96-well Costar microtiter plates. Formula II-2, Formula II-4, Formula II-16, Formula II-17, Formula II-18, Formula II-19, Formula II-21 and Formula II-22 were tested as eight-point dose response curves with final concentrations ranging from 500 nM to 158 pM. Formula II-5A, Formula II-5B and Formula II-20 were tested at concentrations ranging from 1 μ M to 0.32 nM. Formula II-3 was tested as an eight-dose response curve with final concentrations ranging from 10 μ M to 3.2 nM, while Formula II-8C, Formula II-13C, Formula II-24C, Formula II-25 and Formula IV-3C were tested with final concentrations ranging from 20 μ M to 6.3nM. The samples were incubated at 37 °C. for five minutes in a temperature controlled plate reader. During the preincubation step, the substrate was diluted 25-fold in SDS-containing assay buffer. After the preincubation period, the reactions were initiated by the addition of 10 μ l of the diluted substrate and the plates were returned to the plate reader. The final concentration of substrate in the reactions was 20 μ M. All data were collected every five minutes for more than 1.5 hour and plotted as the mean of triplicate data points. The EC₅₀ values (the drug concentration at which 50% of the maximal relative fluorescence unit is inhibited) were calculated by Prism (GraphPad Software) using a sigmoidal dose-response, variable slope model. To evaluate the activity of the compounds against the caspase-like activity of the 20S proteasomes, reactions were performed as described above except that Z-LLE-AMC was used as the

peptide substrate. Formulae II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-17, II-18, II-20, II-21, II-22, II-24C, II-25 and Formula IV-3C were tested at concentrations ranging from 20 μ M to 6.3 nM. Formula II-2 was tested at concentrations ranging from 10 μ M to 3.2 nM, while Formula II-16 and Formula II-19 were tested at concentrations ranging from 5 μ M to 1.58 nM. For the evaluation of the compounds against the trypsin-like activity of the proteasome, the SDS was omitted from the assay buffer and Boc-LRR-AMC was used as the peptide substrate. Formula II-20 was tested at concentrations ranging from 5 μ M to 1.6 nM. Formulae II-3, II-8C, II-13C, II-17, II-21, II-22, II-24C, II-25 and IV-3C were tested at concentrations ranging from 20 μ M to 6.3 nM. For Formulae II-2 and II-5B the concentrations tested ranged from 10 μ M to 3.2 nM, while Formulae II-4, II-5A, II-16, II-18 and II-19 were tested at concentrations ranging from 1 μ M to 0.32 nM.

[0355] Results (mean EC_{50} values) are shown in Table 3 and illustrate that among the tested compounds, Formulae II-5A, II-16, II-18, II-19, II-20, II-21 and II-22 are the most potent inhibitors of the chymotrypsin-like activity of the 20S proteasome with EC_{50} values ranging from 2.2 nM to 7 nM. Formulae II-2, II-4, II-5B and II-17 inhibit the proteasomal chymotrypsin-like activity with EC_{50} values ranging from 14.2 nM to 87 nM, while the EC_{50} value of Formula II-3 is 927 nM. Formula II-24C, II-13C and IV-3C inhibited the chymotrypsin-like activity with EC_{50} values of 2.2 μ M, 8.2 μ M and 7.8 μ M respectively. EC_{50} values for Formulae II-8C and II-25 were greater than 20 μ M. Under the conditions tested, Formulae II-2, II-3, II-4, II-5A, II-5B, II-13C, II-16, II-17, II-18, II-19, II-20, II-21, II-22 and II-24C were able to inhibit the trypsin-like activity of the 20S proteasome. Formulae II-4, II-5A, II-16, II-18 and II-19 inhibited the caspase-like activity with EC_{50} values ranging from 250 nM to 744 nM, while Formulae II-2, II-5B, II-17, II-20, II-21, and II-22 had EC_{50} values ranging from 1.2 μ M to 3.3 μ M.

TABLE 3

Analog	EC_{50} Values		
	Chymotrypsin-like	Trypsin-like	Caspase-like
Formula II-2	18 nM	230 nM	1.5 μ M
Formula II-3	927 nM	6.6 μ M	>20 μ M
Formula II-4	14.2 nM	109 nM	744 nM
Formula II-5A	6.5 nM	89 nM	487 nM
Formula II-5B	87 nM	739 nM	3.3 μ M
Formula II-8C*	>20 μ M	>20 μ M	>20 μ M
Formula II-13C	8.2 μ M	10.7 μ M	>20 μ M
Formula II-16	2.5 nM	21 nM	401 nM
Formula II-17	29.5 nM	588 nM	1.2 μ M
Formula II-18	2.2 nM	14 nM	250 nM
Formula II-19*	3 nM	13 nM	573 nM
Formula II-20*	5 nM	318 nM	1.4 μ M
Formula II-21*	7 nM	720 nM	2.6 μ M
Formula II-22*	5 nM	308 nM	1.3 μ M
Formula II-24C*	2.2 μ M	3.2 μ M	>20 μ M
Formula II-25*	>20 μ M	>20 μ M	>20 μ M
Formula IV-3C	7.8 μ M	>20 μ M	>20 μ M

*n = 1

Example 21

Salinosporamide A (II-16) Inhibits Chymotrypsin-Like Activity of Rabbit Muscle 20S Proteasomes

[0356] The effect of Salinosporamide A (II-16) on proteasomes was examined using a commercially available kit from Calbiochem (catalog no. 539158), which uses a fluorogenic peptide substrate to measure the activity of rabbit muscle 20S proteasomes (Calbiochem 20S Proteasome Kit). This peptide substrate is specific for the chymotrypsin-like enzyme activity of the proteasome.

[0357] Omuralide was prepared as a 10 mM stock in DMSO and stored in 5 μ L aliquots at -80° C. Salinosporamide A was prepared as a 25.5 mM solution in DMSO and stored in aliquots at -80° C. The assay measures the hydrolysis of Suc-LLVY-AMC into Suc-LLVY and AMC. The released coumarin (AMC) was measured fluorometrically by using λ_{ex} =390 nm and λ_{em} =460 nm. The assays were performed in a microtiter plate (Corning 3904), and followed kinetically with measurements every five minutes. The instrument used was a Thermo Lab Systems Fluoroskan, with the incubation chamber set to 37° C. The assays were performed according to the manufacturer's protocol, with the following changes. The proteasome was activated as described with SDS, and held on ice prior to the assay. Salinosporamide A and Omuralide were serially diluted in assay buffer to make an 8-point dose-response curve. Ten microliters of each dose were added in triplicate to the assay plate, and 190 μ L of the activated proteasome was added and mixed. The samples were pre-incubated in the Fluoroskan for 5 minutes at 37° C. Substrate was added and the kinetics of AMC were followed for one hour. All data were collected and plotted as the mean of triplicate data points. The data were normalized to reactions performed in the absence of Salinosporamide A and modeled in Prism as a sigmoidal dose-response, variable slope.

[0358] Similar to the results obtained for the in vitro cytotoxicity (Table 2), Feling, et al., *Angew Chem Int Ed Engl* 42:355 (2003), the EC_{50} values in the 20S proteasome assay showed that Salinosporamide A was approximately 40-fold more potent than Omuralide, with an average value of 1.3 nM versus 49 nM, respectively (FIG. 27). This experiment was repeated and the average EC_{50} in the two assays was determined to be 2 nM for Salinosporamide A and 52 nM for Omuralide.

[0359] Salinosporamide A is a potent inhibitor of the chymotrypsin-like activity of the proteasome. The EC_{50} values for cytotoxicity were in the 10-200 nM range suggesting that the ability of Salinosporamide A to induce cell death was due, at least in large part, to proteasome inhibition. The data suggest that Salinosporamide A is a potent small molecule inhibitor of the proteasome.

Example 22

Salinosporamide A (II-16) Inhibition of PGPH Activity of Rabbit Muscle 20S Proteasomes

[0360] Omuralide can inhibit the PGPH activity (also known as the caspase-like) of the proteasome; therefore, the ability of Salinosporamide A to inhibit the PGPH activity of purified rabbit muscle 20S proteasomes was assessed. A

commercially available fluorogenic substrate specific for the PGPH activity was used instead of the chymotrypsin substrate supplied in the proteasome assay kit described above.

[0361] Salinosporamide A (II-16) was prepared as a 20 mM solution in DMSO and stored in small aliquots at -80°C . The substrate Z-LLE-AMC was prepared as a 20 mM stock solution in DMSO, stored at -20°C . The source of the proteasomes was the commercially available kit from Calbiochem (Cat. #539158). As with the chymotrypsin substrate, the proteasome can cleave Z-LLE-AMC into Z-LLE and free AMC. The activity can then be determined by measuring the fluorescence of the released AMC ($\lambda_{\text{exc}}=390$ nm and $\lambda_{\text{em}}=460$ nm). The proteasomes were activated with SDS and held on ice as per manufacturer's recommendation. Salinosporamide A was diluted in DMSO to generate a 400-fold concentrated 8-point dilution series. The series was diluted 20-fold with assay buffer and preincubated with the proteasomes as described for the chymotrypsin-like activity. After addition of substrate, the samples were incubated at 37°C , and release of the fluorescent AMC was monitored in a fluorimeter. All data were collected and plotted as the mean of triplicate points. In these experiments, the EC_{50} was modeled in Prism as normalized activity, where the amount of AMC released in the absence of Salinosporamide A represents 100% activity. As before, the model chosen was a sigmoidal dose-response, with a variable slope.

[0362] Data revealed that Salinosporamide A inhibited the PGPH activity in rabbit muscle 20S proteasomes with an EC_{50} of 350 nM (FIG. 28). A replicate experiment was performed, which gave a predicted EC_{50} of 610 nM. These results indicate that Salinosporamide A does block the in vitro PGPH activity of purified rabbit muscle 20S proteasomes, albeit with lower potency than seen towards the chymotrypsin-like activity.

Example 23

Inhibition of the Chymotrypsin-Like Activity of Human Erythrocyte 20S Proteasomes

[0363] The ability of Salinosporamide A (II-16) to inhibit the chymotrypsin-like activity of human erythrocyte 20S proteasomes was assessed in vitro. The calculated EC_{50} values ranged from 45 to 247 pM, and seemed to depend upon the lot of proteasomes tested (BIOMOL, Cat#SE-221). These data indicate that the inhibitory effect of Salinosporamide A is not limited to rabbit skeletal muscle proteasomes.

[0364] Salinosporamide A was prepared as a 20 mM solution in DMSO and stored in small aliquots at -80°C . The substrate, suc-LLVY-AMC, was prepared as a 20 mM solution in DMSO and stored at -20°C . Human erythrocyte 20S proteasomes were obtained from BIOMOL (Cat. # SE-221). The proteasome can cleave suc-LLVY-AMC into suc-LLVY and free AMC and the activity can then be determined by measuring the fluorescence of the released AMC ($\lambda_{\text{exc}}=390$ nm and $\lambda_{\text{em}}=460$ nm). The proteasomes were activated by SDS and stored on ice as with the experiments using rabbit muscle proteasomes. Salinosporamide A was diluted in DMSO to generate a 400-fold concentrated 8-point dilution series. The series was then diluted 20-fold with assay buffer and pre-incubated with proteasomes at 37°C . The reaction was initiated with substrate, and the release of AMC was followed in a

Fluoroskan microplate fluorimeter. Data were collected and plotted as the mean of triplicate points. Data were captured kinetically for 3 hours, and indicated that these reactions showed linear kinetics in this time regime. The data were normalized to reactions performed in the absence of Salinosporamide A and modeled in Prism as a sigmoidal dose-response, variable slope.

[0365] Replicate experiments performed using human erythrocyte proteasomes from separate lots resulted in a range of EC_{50} values between 45 and 250 pM (FIG. 29 shows a representative experiment). It has been reported that 20S proteasomes purified from human erythrocytes are highly heterogeneous in subunit composition. Claverol, et al., *Mol Cell Proteomics* 1:567 (2002). The variability in these experiments may therefore be due to differences in the composition and activity of the human erythrocyte proteasome preparations. Regardless, these results indicate that the in vitro chymotrypsin-like activity of human erythrocyte 20S proteasomes is sensitive to Salinosporamide A.

Example 24

Salinosporamide A (II-16) Specificity

[0366] A possible mechanism by which Salinosporamide A inhibits the proteasome is by the reaction of the β -lactone functionality of Salinosporamide A with the active site threonine of the proteasome. This covalent modification of the proteasome would block the active site, as this residue is essential for the catalytic activity of the proteasome. Fenteany, et al., *J Biol Chem* 273:8545 (1998). A structurally related compound, Lactacystin, has been shown to also inhibit cathepsin A (Ostrowska, et al., *Int J Biochem Cell Biol* 32:747 (2000), Kozłowski, et al., *Tumour Biol* 22:211 (2001), Ostrowska, et al., *Biochem Biophys Res Commun* 234:729 (1997)) and TPPII (Geier, et al., *Science* 283:978 (1999)) but not trypsin, chymotrypsin, papain, calpain (Fenteany, et al., *Science* 268:726 (1995)), thrombin, or plasminogen activator (Omura, et al., *J Antibiot* (Tokyo) 44:113 (1991)). Similar studies were initiated to explore the specificity of Salinosporamide A for the proteasome by evaluating its ability to inhibit the catalytic activity of a prototypical serine protease, chymotrypsin.

[0367] Salinosporamide A was prepared as a 20 mM solution in DMSO and stored in small aliquots at -80°C . The substrate, suc-LLVY-AMC, was prepared as a 20 mM solution in DMSO and stored at -20°C . Proteolytic cleavage of this substrate by either proteasomes or chymotrypsin liberates the fluorescent product AMC, which can be monitored in a fluorimeter ($\lambda_{\text{exc}}=390$ nm and $\lambda_{\text{em}}=460$ nm). Bovine pancreatic chymotrypsin was obtained from Sigma (Cat. #C-4129), and prepared as a 5 mg/ml solution in assay buffer (10 mM HEPES, 0.5 mM EDTA, 0.05% Triton X-100, pH 7.5) daily. Immediately prior to the assay, the chymotrypsin was diluted to 1 $\mu\text{g}/\text{ml}$ (0.2 $\mu\text{g}/\text{well}$) in assay buffer and held on ice. Salinosporamide A was diluted in DMSO to generate an 8-point dose-response curve. The high final Salinosporamide A concentrations needed to obtain complete inhibition of chymotrypsin required that the diluted enzyme be directly added to the compound dilution series. The inclusion of 1% DMSO (the final concentration of solvent in the test wells) into the reaction had no significant effect on chymotrypsin activity towards this substrate. The reactions were pre-incubated for 5 minutes at 37°C and

the reactions were initiated by the addition of substrate. Data were collected kinetically for one hour at 37° C. in the Fluoroskan and plotted as the mean of triplicate data points. The data were normalized to reactions performed in the absence of Salinosporamide A, and modeled in Prism as a sigmoidal dose-response, variable slope. Normalized data from Salinosporamide A inhibition of the chymotrypsin-like activity of rabbit 20S proteasomes has been included on the same graph.

[0368] The average inhibition observed in two experiments using Salinosporamide A pretreatment of chymotrypsin was 17.5 μ M (FIG. 30 shows a representative experiment). The data indicate that there is a preference for Salinosporamide A-mediated inhibition of the *in vitro* chymotrypsin-like activity of proteasomes over inhibition of the catalytic activity of chymotrypsin.

[0369] Thus, Salinosporamide A inhibits the chymotrypsin-like and PGPH activity of the proteasome. Preliminary studies indicate that Salinosporamide A also inhibits the trypsin-like activity of the proteasome with an EC₅₀ value of ~10 nM (data not shown).

Example 25

Inhibition of NF- κ B-Mediated Luciferase Activity by Formulae II-2, II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-16, II-17, II-18, II-19, II-20, II-21, II-22, II-24C, II-25 and IV-3C; HEK293 NF- κ B/Luciferase Reporter Cell Line

[0370] The HEK293 NF- κ B/luciferase reporter cell line is a derivative of the human embryonic kidney cell line (ATCC; CRL-1573) and carries a luciferase reporter gene under the regulation of 5xNF- κ B binding sites. The reporter cell line was routinely maintained in complete DMEM medium (DMEM plus 10% (v/v) Fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES and Penicillin/Streptomycin at 100 IU/ml and 100 μ g/ml, respectively) supplemented with 250 μ g/ml G418. When performing the luciferase assay, the DMEM basal medium was replaced with phenol-free DMEM basal medium and the G418 was omitted. The cells were cultured in an incubator at 37° C. in 5% CO₂ and 95% humidified air.

[0371] For NF- κ B-mediated luciferase assays, HEK293 NF- κ B/luciferase cells were seeded at 1.5×10^4 cells/well in 96 μ l phenol-free DMEM complete medium into Corning 3917 white opaque-bottom tissue culture plates. For Formula II-2, Formula II-4, Formula II-5A, Formula II-16 and Formula II-18, a 400 μ M starting dilution was made in 100% DMSO and this dilution was used to generate a 8-point half log dilution series. This dilution series was further diluted 40x in appropriate culture medium and ten μ l aliquots were added to the test wells in triplicate resulting in final test concentrations ranging from 1 μ M to 320 μ M. For Formula II-3, Formula II-5B, Formula II-8C, Formula II-13C, Formula II-17, Formula II-20, Formula II-21, Formula II-22, Formula II-24C, Formula II-25 and IV-3C, a 8 mM starting dilution was made in 100% DMSO and the same procedure was followed as described above resulting in final test concentrations ranging from 20 μ M to 6.3 nM. For Formula II-19, a 127 μ M starting dilution was made in 100% DMSO and the final test concentrations ranging from 317 nM to 0.1 nM. The plates were returned to the incubator

for 1 hour. After 1 hr pretreatment, 10 μ l of a 50 ng/ml TNF- α solution, prepared in the phenol-free DMEM medium was added, and the plates were incubated for an additional 6 hr. The final concentration of DMSO was 0.25% in all samples.

[0372] At the end of the TNF- α stimulation, 100 μ l of Steady Lite HTS luciferase reagent (Packard Bioscience) was added to each well and the plates were left undisturbed for 10 min at room temperature before measuring the luciferase activity. The relative luciferase units (RLU) were measured by using a Fusion microplate fluorometer (Packard Bioscience). The EC₅₀ values (the drug concentration at which 50% of the maximal relative luciferase unit inhibition is established) were calculated in Prism (GraphPad Software) using a sigmoidal dose response, variable slope model.

[0373] Inhibition of NF- κ B Activation by Formulae II-2, II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-16, II-17, II-18, II-19, II-20, II-21, II-22, II-24C, II-25 and IV-3C

[0374] NF- κ B regulates the expression of a large number of genes important in inflammation, apoptosis, tumorigenesis, and autoimmune diseases. In its inactive form, NF- κ B complexes with I κ B in the cytosol and upon stimulation, I κ B is phosphorylated, ubiquitinated and subsequently degraded by the proteasome. The degradation of I κ B leads to the activation of NF- κ B and its translocation to the nucleus. The effects of Formula II-2, Formula II-3, Formula II-4, Formula II-5A, Formula II-5B, Formula II-8C, Formula II-13C, Formula II-16, Formula II-17, Formula II-18, Formula II-19, Formula II-20, Formula II-21, Formula II-22, Formula II-24C, Formula II-25 and Formula IV-3C on the activation of NF- κ B were evaluated by assessing the NF- κ B-mediated luciferase activity in HEK293 NF- κ B/Luc cells upon TNF- α stimulation.

[0375] Pretreatment of NF- κ B/Luc 293 cells with Formula II-2, Formula II-4, Formula II-5A, Formula II-5B, Formula II-16, Formula II-17, Formula II-18, Formula II-19, Formula II-20, Formula II-21, Formula II-22 and Formula II-24C resulted in a dose-dependent decrease of luciferase activity upon TNF- α stimulation. The mean EC₅₀ values to inhibit NF- κ B-mediated luciferase activity are shown in Table 4 and demonstrate that compounds of Formula II-2, Formula II-4, Formula II-5A, Formula II-5B, Formula II-16, Formula II-17, Formula II-18, Formula II-19, Formula II-20, Formula II-21, Formula II-22 and Formula II-24C inhibited NF- κ B activity in this cell-based assay.

TABLE 4

Mean EC ₅₀ values of Formula II-2, II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-16, II-17, II-18, II-19, II-20, II-21, II-22, II-24C, II-25 and IV-3C from NF- κ B-mediated luciferase reporter gene assay	
Compound	EC ₅₀ (nM)
Formula II-2	82
Formula II-3	>20,000
Formula II-4	77.7
Formula II-5A	31.5
Formula II-5B	270
Formula II-8C*	>20,000
Formula II-13C	>20,000
Formula II-16	11.8
Formula II-17	876
Formula II-18	9.5

TABLE 4-continued

Mean EC ₅₀ values of Formulas II-2, II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-16, II-17, II-18, II-19, II-20, II-21, II-22, II-24C, II-25 and IV-3C from NF- κ B-mediated luciferase reporter gene assay	
Compound	EC ₅₀ (nM)
Formula II-19	8.5
Formula II-20*	154
Formula II-21*	3,172
Formula II-22*	1,046
Formula II-24C*	5,298
Formula II-25*	>20,000
Formula IV-3C	>20,000

*n = 1

Example 26

Effect of Salinosporamide A on the NF- κ B Signaling Pathway

[0376] Experiments were carried out to study the role of Salinosporamide A in the NF- κ B signaling pathway. A stable HEK293 clone (NF- κ B/Luc 293) was generated carrying a luciferase reporter gene under the regulation of 5xNF- κ B binding sites. Stimulation of this cell line with TNF- α leads to increased luciferase activity as a result of NF- κ B activation.

[0377] NF- κ B/Luc 293 cells were pre-treated with 8-point half-log serial dilutions of Salinosporamide A (ranging from 1 μ M to 317 pM) for 1 hour followed by a 6 hour stimulation with TNF- α (10 ng/mL). NF- κ B inducible luciferase activity was measured at 6 hours. Viability of NF- κ B/Luc 293 cells, after treatment with Salinosporamide A for 24 hr, was assessed by the addition of resazurin dye, as previously described.

[0378] Pretreatment of NF- κ B/Luc 293 cells with Salinosporamide A resulted in a dose-dependent decrease of luciferase activity upon TNF- α stimulation (FIG. 31, right y-axis). The calculated EC₅₀ for inhibition of NF- κ B/luciferase activity was ~7 nM. A cytotoxicity assay was simultaneously performed, and showed that this concentration of Salinosporamide A did not affect cell viability (FIG. 31, left y-axis). These representative data suggested that the observed decrease in luciferase activity by Salinosporamide A treatment was primarily due to an NF- κ B mediated-signaling event rather than cell death.

Example 27

[0379] In addition to the NF- κ B luciferase reporter gene assay, the effect of Salinosporamide A on the levels of phosphorylated-I κ B α and total I κ B α was evaluated by western blot. Endogenous protein levels were assessed in both HEK293 cells and the NF- κ B/Luc 293 reporter clone.

[0380] Cells were pre-treated for 1 hour with Salinosporamide A at the indicated concentrations followed by stimulation with 10 ng/mL of TNF- α for 30 minutes. Antibodies against total and phosphorylated forms of I κ B α were used to determine the endogenous level of each protein and anti-Tubulin antibody was used to confirm equal loading of protein.

[0381] As shown in FIG. 32, treatment of both cell lines with Salinosporamide A at 50 and 500 nM not only reduced

the degradation of total I κ B α but also retained the phospho-I κ B α level when stimulated with TNF- α . These results strongly support the mechanism of action of Salinosporamide A as a proteasome inhibitor, which prevents the degradation of phosphorylated I κ B α upon TNF- α stimulation.

Example 28

Effect of Salinosporamide A on Cell Cycle Regulatory Proteins

[0382] The ubiquitin-proteasome pathway is an essential proteolytic system involved in cell cycle control by regulating the degradation of cyclins and cyclin-dependent kinase (Cdk) inhibitors such as p21 and p27. Pagano, et al., *Science* 269:682 (1995), Kisselev, et al., *Chem Biol* 8:739 (2001), King, et al., *Science* 274:1652 (1996). Furthermore, p21 and p27 protein levels are increased in the presence of proteasome inhibitors. Fukuchi, et al., *Biochim Biophys Acta* 1451:206 (1999), Takeuchi, et al., *Jpn J Cancer Res* 93:774 (2002). Therefore, western blot analysis was performed to evaluate the effect of Salinosporamide A treatment on endogenous levels of p21 and p27 using the HEK293 cells and the HEK293 NF- κ B/Luciferase reporter clone.

[0383] The Western blots presented in FIG. 33 were probed using antibodies against p21 and p27 to determine the endogenous level of each protein and anti-Tubulin antibody was used to confirm equal loading of protein.

[0384] As shown in FIGS. 33A and 33B, preliminary results indicated that p21 and p27 protein levels were elevated when both cell lines were treated with Salinosporamide A at various concentrations. Data showed that Salinosporamide A acts by inhibiting proteasome activity thereby preventing the TNF- α induced activation of NF- κ B. In addition, this proteasomal inhibition results in the accumulation of the Cdk inhibitors, p21 and p27, which has been reported to sensitize cells to apoptosis. Pagano, et al., *supra* (1995), King, et al., *supra* (1996).

Example 29

Activation of Caspase-3 by Salinosporamide A (II-16)

[0385] To address whether Salinosporamide A induces apoptosis, its effect on the induction of Caspase-3 activity was evaluated using Jurkat cells (American Type Culture Collection (ATCC) TIB-152, human acute T cell leukemia).

[0386] Jurkat cells were plated at 2×10^6 cells/3 mL per well in a 6-well plate and incubated at 37°C, 5% (v/v) CO₂ and 95% (v/v) humidity. Salinosporamide A and Mitoxantrone (Sigma, St. Louis, Mo. Cat #M6545), were prepared in DMSO at stock concentrations of 20 mM and 40 mM, respectively. Mitoxantrone is a chemotherapeutic drug that induces apoptosis in dividing and non-dividing cells via inhibition of DNA synthesis and repair and was included as a positive control. Bhalla, et al., *Blood* 82:3133 (1993). Cells were treated with EC₅₀ concentrations (Table 5) and incubated 19 hours prior to assessing of Caspase-3 activity. Cells treated with 0.25% DMSO served as the negative control. The cells were collected by centrifugation and the media removed. Cell pellets were processed for the Caspase-3 activity assay as described in the manufacturer's protocol (EnzChek Caspase-3 Assay Kit from Molecular Probes

(E-13183; see Appendix G, which form a part of this application and is also available at hypertext transfer protocol on the worldwide web at "probes.com/media/pis/mp13183.pdf." In brief, cell pellets were lysed on ice, mixed with the EnzChek Caspase-3 components in a 96-well plate, and then incubated in the dark for 30 minutes prior to reading fluorescence of cleaved benzyloxycarbonyl-DEVD-AMC using a Packard Fusion with $\lambda_{ex}=485$ nm and $\lambda_{em}=530$ nm filters. Protein concentrations for lysates were determined using the BCA Protein Assay Kit (Pierce) and these values were used for normalization.

[0387] Data from representative experiments indicate that Salinosporamide A treatment of Jurkat cells results in cytotoxicity and activation of Caspase-3 (Table 5, FIG. 34).

TABLE 5

EC50 Values of Salinosporamide A and Mitoxantrone Cytotoxicity against Jurkat Cells		
Jurkat Cells		
Compound	EC ₅₀ (nM)	% max cell kill
Salinosporamide A	10	97
Mitoxantrone	50	99

Example 30

PARP Cleavage by Salinosporamide A in Jurkat Cells

[0388] In order to assess the ability of Salinosporamide A to induce apoptosis in Jurkat cells, cleavage of poly(ADP-ribose) polymerase (PARP) was monitored. PARP is a 116 kDa nuclear protein that is one of the main intracellular targets of Caspase-3. Decker, et al., *J Biol Chem* 275:9043 (2000), Nicholson, D. W., *Nat Biotechnol* 14:297 (1996). The cleavage of PARP generates a stable 89 kDa product, and this process can be monitored by western blotting. Cleavage of PARP by caspases is a hallmark of apoptosis, and as such serves as an excellent marker for this process.

[0389] Jurkat cells were maintained in RPMI supplemented with 10% Fetal Bovine Serum (FBS) at low density (2×10^5 cells per mL) prior to the experiment. Cells were harvested by centrifugation, and resuspended in media to 1×10^6 cells per 3 mL. Twenty mL of the cell suspension were treated with 100 nM Salinosporamide A (20 mM DMSO stock stored at -80°C), and a 3 mL aliquot removed and placed on ice for the T₀ sample. Three mL aliquots of the cell suspension plus Salinosporamide A were placed in 6-well dishes and returned to the incubator. As a positive control for PARP cleavage, an identical cell suspension was treated with 350 nM Staurosporine, a known apoptosis inducer (Sigma S5921, 700 μM DMSO stock stored at -20°C). Samples were removed at 2, 4, 6, 8, and 24 hrs in the case of Salinosporamide A treated cells, and at 4 hrs for the Staurosporine control. For each time point, the cells were recovered by brief centrifugation, the cells were washed with 400 μL of PBS, and the cells pelleted again. After removal of the PBS, the pellets were stored at -20°C prior to SDS PAGE. Each cell pellet was resuspended in 100 μL of NuPAGE sample buffer (Invitrogen 46-5030) and 10 μL of each sample were separated on 10% NuPAGE BIS-Tris gels

(Invitrogen NB302). After electrotransfer to nitrocellulose, the membrane was probed with a rabbit polyclonal antibody to PARP (Cell Signaling 9542), followed by goat anti-rabbit alkaline phosphatase conjugated secondary antibody (Jackson 11-055-045). Bound antibodies were detected colorimetrically using BCIP/NBT (Roche 1681451).

[0390] The western blot presented in FIG. 35 shows the cleavage of PARP within the Jurkat cells in a time-dependent fashion. The cleaved form (denoted by the asterisk, *) appears in the treated cells between 2 and 4 hrs after exposure to Salinosporamide A while the majority of the remaining PARP is cleaved by 24 hrs. The Staurosporine treated cells (St) show rapid cleavage of PARP with most of this protein being cleaved within 4 hours. These data strongly suggest that Salinosporamide A can induce apoptosis in Jurkat cells.

Example 31

Anti-Anthrax Activity

[0391] In order to assay for the ability of Salinosporamide A or other compounds to prevent cell death resulting from LeTx exposure, RAW264.7 macrophage-like cells and recombinant LF and PA lethal toxin components were used as an in vitro model system assaying for cytotoxicity, as described below.

[0392] RAW264.7 cells (ATCC #ITB-71) were adapted to and maintained in Advanced Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, Calif.) supplemented with 5% fetal bovine serum (ADMEM, Mediatech, Herndon, Va.) at 37°C in a humidified 5% CO₂ incubator. Cells were plated overnight in ADMEM supplemented with 5% FBS at 37°C in a humidified 5% CO₂ incubator at a concentration of 50,000 cells/well in a 96-well plate. Alternatively, cells cultured in DMEM supplemented with 10% fetal calf serum were also used and found to be amenable to this assay. Media was removed the following morning and replaced with serum-free ADMEM with or without Salinosporamide A or Omuralide at doses ranging from 1 μM to 0.5 nM for an 8-point dose-response. The compounds were prepared from a 1 mg/mL DMSO stock solution and diluted to the final concentration in ADMEM. After a 15 minute pre-incubation, 200 ng/mL LF or 400 ng/mL PA alone or in combination (LeTx) were added to cells. Recombinant LF and PA were obtained from List Biological Laboratories and stored as 1 mg/mL stock solutions in sterile water containing 1 mg/mL BSA at -80°C as described by the manufacturer. Cells were incubated for 6 hours at 37°C , followed by addition of Resazurin as previously described. Plates were incubated an additional 6 hours prior to assessing cell viability by measuring fluorescence. The data are a summary of three experiments with three to six replicates per experiment and are expressed as the percent viability using the DMSO (negative) and the LeTx controls (positive) to normalize the data using the following equation: % viability = $100 \times (\text{observed OD-positive control}) / (\text{negative control-positive control})$.

[0393] The data represented in FIG. 36 indicate that treatment with Salinosporamide A can prevent LeTx-induced cell death of macrophage-like RAW264.7 cells in vitro. Treatment of RAW cells with either LF or PA alone or Salinosporamide A alone resulted in little reduction in cell

viability, whereas treatment with LeTx resulted in approximately 0.27% cell viability as compared to controls. Salinosporamide A may enhance macrophage survival by inhibiting the degradation of specific proteins and decreasing the synthesis of cytokines, which will ultimately lead to the inhibition of the lethal effects of anthrax toxins *in vivo*.

[0397] EC₅₀ values were determined in standard growth inhibition assays using Resazurin dye and 48 hour of drug exposure. Results from 2-5 independent experiments (Table 6) show that the EC₅₀ values for Salinosporamide A against RPMI 8226 and the prostate cell lines range from 10-37 nM.

TABLE 6

Compound	EC ₅₀ values of Salinosporamide A (II-16) against Multiple Myeloma and Prostate Tumor cell lines					
	RPMI 8226 (n = 5)			DU 145 (n = 3)		
	EC ₅₀		%	EC ₅₀		%
	(nM), mean ± SD	cytotoxicity, mean ± SD		(nM), mean ± SD	cytotoxicity, mean ± SD	
Salinosporamide A	10 ± 3	94 ± 1		37 ± 10	75 ± 4	31, 25, 88, 89

[0394] Although Salinosporamide A treatment alone produced very modest cytotoxicity at concentrations of 100 nM and above, treatment with lower, relatively non-toxic levels revealed a marked increase in RAW 264.7 cell viability in LeTx treated cells (FIG. 36). For example, the Salinosporamide A+LeTx treated group showed 82% cell-viability when pretreated with 12 nM Salinosporamide A, which was a concentration that showed 96% viability with Salinosporamide A alone. The average EC₅₀ for Salinosporamide A in these studies was 3.6 nM. In contrast, Omuralide showed relatively little effect on cell viability until concentrations of 1 μM were reached. Even at this high concentration of Omuralide, only 37% viability was observed indicating that Salinosporamide A is a more potent inhibitor of LeTx-induced RAW264.7 cell death. Consistent with these data, Tang et. al., *Infect Immun* 67:3055 (1999), found that the EC₅₀ concentrations for MG132 and Lactacystin (the precursor to Omuralide) in the LeTx assay were 3 μM. Taken together, these data further illustrate that Salinosporamide A is a more potent inhibitor of LeTx-induced cytotoxicity than any other compound described to date.

[0395] Salinosporamide A promoted survival of RAW264.7 cells in the presence of LeTx indicating that this compound or it's derivatives may be a valuable clinical therapeutic for anthrax. In addition, it is worth noting that Salinosporamide A is much less cytotoxic on RAW 264.7 cells than for many tumor cells.

Example 32

Activity of Salinosporamide A Against Multiple Myeloma and Prostate Cancer Cell Lines

[0396] NF-κB appears to be critical to the growth and resistance to apoptosis in Multiple Myeloma and has also been reported to be constitutively active in various prostate cancer cell lines (Hideshima T et al. 2002, Shimada K et al. 2002 and Palayoor S T et al. 1999). NF-κB activity is regulated by the proteasomal degradation of its inhibitor IκBα. Since Salinosporamide A has been shown to inhibit the proteasome *in vitro* and to interfere with the NF-κB signaling pathway, the activity of Salinosporamide A against the multiple myeloma cell line RPMI 8226 and the prostate cancer cell lines PC-3 and DU 145 was evaluated.

[0398] The ability of Salinosporamide A to induce apoptosis in RPMI 8226 and PC-3 cells was evaluated by monitoring the cleavage of PARP and Pro-Caspase 3 using western blot analysis. Briefly, PC-3 and RPMI 8226 cells were treated with 100 nM Salinosporamide A (2345R01) for 0, 8 or 24 hours. Total protein lysates were made and 20 μg of the lysates were then resolved under reducing/denaturing conditions and blotted onto nitrocellulose. The blots were then probed with anti-PARP or anti-caspase 3 antibodies followed by stripping and reprobing with an anti-actin antibody.

[0399] Results of these experiments illustrate that Salinosporamide A treatment of RPMI 8226 cells leads to the cleavage of PARP and Pro-caspase 3 in a time-dependent manner (FIG. 37). RPMI 8226 cells seem to be more sensitive to Salinosporamide A than PC-3 cells since the induction of PARP cleavage is already noticeable at 8 hours and complete by 24 hours. In contrast, in PC-3 cells the cleavage of PARP is noticeable at 24 hours, while the cleavage of Pro-Caspase 3 is not detected in this experiment (FIG. 37).

[0400] RPMI 8226 cells were used to evaluate the effect of treating the cells for 8 hours with various concentrations of Salinosporamide A. Briefly, RPMI 8226 cells were treated with varying concentrations of Salinosporamide A (2345R01) for 8 hours and protein lysates were made. 25 μg of the lysates were then resolved under reducing/denaturing conditions and blotted onto nitrocellulose. The blots were then probed with anti-PARP or anti-caspase 3 antibodies followed by stripping and reprobing with an anti-actin antibody. FIG. 38 demonstrates that Salinosporamide A induces a dose-dependent cleavage of both PARP and Pro-Caspase 3.

Example 33

Growth Inhibition of Human Multiple Myeloma by Formulas II-2, II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-16, II-17, II-18, II-19, II-20, and IV-3C; RPMI 8226 and U266 Cells

[0401] The human multiple myeloma cell lines, RPMI 8226 (ATCC; CCL-155) and U266 (ATCC; TIB-196) were

maintained in appropriate culture media. The cells were cultured in an incubator at 37° C. in 5% CO₂ and 95% humidified air.

[0402] For cell growth inhibition assays, RPMI 8226 cells and U266 were seeded at 2×10⁴ and 2.5×10⁴ cells/well respectively in 90 μ l complete media into Corning 3904 black-walled, clear-bottom tissue culture plates. 20 mM stock solutions of the compounds were prepared in 100% DMSO, aliquoted and stored at -80° C. The compounds were serially diluted and added in triplicate to the test wells. The final concentration range of Formula II-3, II-8C, II-5B, II-13C, II-17, IV-3C and II-20 were from 20 μ M to 6.32 nM. The final concentration of Formula II-16, II-18 and II-19 ranged from 632 nM to 200 pM. The final concentration

(generated by XLfit 3.0, ID Business Solutions Ltd). The data are summarized in Tables 13 and 15.

Example 34

Salinosporamide A (II-16) Retains Activity Against the Multi-Drug Resistant Cell Lines MES-SA/Dx5 and HL-60/MX2

[0404] The EC₅₀ values of Salinosporamide A against the human uterine sarcoma MES-SA cell line and its multidrug-resistant derivative MES-SA/Dx5 were determined to evaluate whether Salinosporamide A retains activity against a cell line overexpressing the P-glycoprotein efflux pump. Paclitaxel, a known substrate for the P-glycoprotein pump was included as a control.

TABLE 7

EC₅₀ values of Salinosporamide A against MES-SA and the drug-resistant derivative MES-SA/Dx5

	MES-SA		MES-SA/Dx5		Fold change
	EC ₅₀ (nM), mean	% cytotoxicity, mean \pm SD	EC ₅₀ (nM), mean \pm SD	% cytotoxicity, mean \pm SD	
Salinosporamide A	20 \pm 5	94 \pm 1	23 \pm 1	92 \pm 2	1.2
Paclitaxel	5 \pm 2	63 \pm 7	2040 \pm 150	78 \pm 1	408

range of Formula II-2, II-4 and II-5A were from 2 μ M to 632 pM. The final concentration of DMSO was 0.25% in all samples.

[0403] Following 48 hours of drug exposure, 10 μ l of 0.2 mg/ml resazurin (obtained from Sigma-Aldrich Chemical Co.) in Mg²⁺, Ca²⁺ free phosphate buffered saline was added to each well and the plates were returned to the incubator for 3-6 hours. Since living cells metabolize Resazurin, the fluorescence of the reduction product of Resazurin was measured using a Fusion microplate fluorometer (Packard Bioscience) with λ_{ex} =535 nm and λ_{em} =590 nm filters. Resazurin dye in medium without cells was used to determine the background, which was subtracted from the data for all experimental wells. The data were normalized to the average fluorescence of the cells treated with media +0.25% DMSO (100% cell growth) and EC₅₀ values (the drug concentration at which 50% of the maximal observed

[0405] Results from these growth inhibition assays (Table 7) show that, as expected, Paclitaxel did not retain its activity against MES-SA/Dx5 cells as reflected by the 408 fold increase in the EC₅₀ values. EC₅₀ values for Salinosporamide A against MES-SA and MES-SA/Dx5 were similar. This illustrates that Salinosporamide A is able to inhibit the growth of the multi-drug resistant cell line MES-SA/Dx5 suggesting that Salinosporamide A does not seem to be a substrate for the P-glycoprotein efflux pump.

[0406] In addition, Salinosporamide A was evaluated against HL-60/MX2, the drug resistant derivative of the human leukemia cell line, HL-60, characterized by having a reduced Topoisomerase II activity and considered to have atypical multidrug resistance. EC₅₀ values for growth inhibition were determined for Salinosporamide A against the HL-60 and HL-60/MX2. The DNA binding agent Mitoxantrone was included as a control, as HL-60/MX2 cells are reported to be resistant to this chemotherapeutic agent (Harker W. G. et al. 1989).

TABLE 8

EC₅₀ values of Salinosporamide A against HL-60 and the drug resistant derivative HL-60/MX2

	HL-60		HL-60/MX2		Fold change
	EC ₅₀ (nM)	% cytotoxicity	EC ₅₀ (nM)	% cytotoxicity	
Salinosporamide A	27, 30	88, 91	28, 25	84, 89	1.0, 0.8
Mitoxantrone	59, 25	98, 100	1410, 827	98, 99	24, 33

growth inhibition is established) were determined using a standard sigmoidal dose response curve fitting algorithm

[0407] The data in Table 8 reveals that Salinosporamide A was able to retain its activity against HL-60/MX2 cells

relative to HL-60 cells, indicating that Salinosporamide A is active in cells expressing reduced Topoisomerase II activity. In contrast, Mitoxantrone was about 29 fold less active against HL-60/MX2 cells.

Example 35

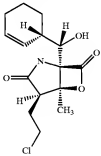
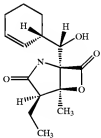
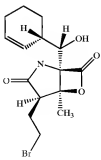
Salinosporamide A and Several Analogs: Structure Activity Relationship

[0408] To establish an initial structure activity relationship (SAR) for Salinosporamide A, a series of Salinosporamide A analogs were evaluated against the multiple myeloma cell line RPMI 8226. EC_{50} values were determined in standard growth inhibition assays using Resazurin dye and 48 hour of drug exposure.

[0409] The results of this initial series of SAR (Table 9) indicate that the addition of a halogen group to the ethyl group seems to enhance the cytotoxic activity.

TABLE 9

Initial SAR series of Salinosporamide A

Compound No.	Molecular Structure	EC_{50} (μ M (mean \pm SD))	% Cytotoxicity (mean \pm SD)
II-16		0.007 \pm 0.0001	94 \pm 0
II-17		2.6, 2.3	94, 95
II-18		0.017, 0.022	94, 94

[0410] Where $n > 2$, mean \pm standard deviation was determined

Example 36

In vivo Biology

Maximum Tolerated Dose (MTD) Determination

[0411] In vivo studies were designed to determine the MTD of Salinosporamide A when administered intravenously to female BALB/c mice.

[0412] BALB/c mice were weighed and various Salinosporamide A concentrations (ranging from 0.01 mg/kg to 0.5 mg/kg) were administered intravenously as a single dose (qdx1) or daily for five consecutive days (qdx5). Animals were observed daily for clinical signs and were weighed individually twice weekly until the end of the experiment (maximum of 14 days after the last day of dosing). Results are shown in Table 11 and indicate that a single intravenous Salinosporamide A dose of up to 0.25 mg/kg was tolerated. When administered daily for five consecutive days, concentrations of Salinosporamide A up to 0.1 mg/kg were well tolerated. No behavioral changes were noted during the course of the experiment.

TABLE 11

MTD Determination of Salinosporamide A in female BALB/c Mice

Group	Dose (mg/kg)	Route/Schedule	Deaths/Total	Days of Deaths
1	0.5	I.v.; qdx1	3/3	3, 3, 4
2	0.25	I.v.; qdx1	0/3	
3	0.1	I.v.; qdx1	0/3	
4	0.05	I.v.; qdx1	0/3	
5	0.01	I.v.; qdx1	0/3	
6	0	I.v.; qdx1	0/3	
7	0.5	I.v.; qdx5	3/3	4, 6, 7
8	0.25	I.v.; qdx5	3/3	4, 5, 5
9	0.1	I.v.; qdx5	0/3	
10	0.05	I.v.; qdx5	0/3	
11	0.01	I.v.; qdx5	0/3	
12	0	I.v.; qdx5	0/3	

Example 37

Preliminary Assessment of Salinosporamide A Absorption, Distribution, Metabolism and Elimination (ADME) Characteristics

[0413] Studies to initiate the evaluation of the ADME properties of Salinosporamide A were performed. These studies consisted of solubility assessment, $LogD^{7.4}$ determination and a preliminary screen to detect cytochrome P450 enzyme inhibition. Results from these studies showed an estimated solubility of Salinosporamide A in PBS (pH 7.4) of 9.6 μ M (3 μ g/ml) and a $LogD^{7.4}$ value of 2.4. This $LogD^{7.4}$ value is within the accepted limits compatible with drug development ($LogD^{7.4} < 5.0$) and suggests oral availability. Results from the preliminary P450 inhibition screen showed that Salinosporamide A, when tested at 10 μ M, showed no or low inhibition of all P450 isoforms: CYP1A2, CYP2C9 and CYP3A4 were inhibited by 3%, 6% and 6% respectively, while CYP2D6 and CYP2C19 were inhibited by 19% and 22% respectively.

Example 38

Salinosporamide A and its Effects *in vivo* on Whole Blood Proteasome Activity

[0414] Salinosporamide A was previously demonstrated to be a potent and specific inhibitor of the proteasome *in vitro*, with an IC_{50} of 2 nM towards the chymotrypsin-like activity of purified 20S proteasomes. To monitor the activity of Salinosporamide A *in vivo*, a rapid and reproducible assay (adapted from Lightcap et al. 2000) was developed to assess the proteasome activity in whole blood.

[0415] In brief, frozen whole blood samples were thawed on ice for one hour, and resuspended in 700 μ L of ice cold 5 mM EDTA, pH 8.0 in order to lyse the cells by hypotonic shock. This represents approximately 2-3 times the volume of the packed whole blood cells. Lysis was allowed to proceed for one hour, and the cellular debris was removed by centrifugation at 14,000g for 10 minutes. The supernatant (Packed Whole Blood Lysate, PWBL) was transferred to a fresh tube, and the pellet discarded. Protein concentration of the PWBL was determined by BCA assay (Pierce) using BSA as a standard. Approximately 80% of the samples had a total protein concentration between 800 and 1200 μ g/mL.

[0416] Proteasome activity was determined by measuring the hydrolysis of a fluorogenic substrate specific for the chymotrypsin-like activity of proteasomes (suc-LLVY-AMC, Bachem Cat. 1-1395). Control experiments indicated that >98% of the hydrolysis of this peptide in these extracts is mediated by the proteasome. Assays were set up by mixing 5 μ L of a PWBL from an animal with 185 μ L of assay buffer (20 mM HEPES, 0.5 mM EDTA, 0.05% Triton X-100, 0.05% SDS, pH 7.3) in Costar 3904 plates. Titration experiments revealed there is a linear relationship between protein concentration and hydrolysis rate if the protein concentration in the assay is between 200 and 1000 μ g. The reactions were initiated by the addition of 10 μ L of 0.4 mM suc-LLVY-AMC (prepared by diluting a 10 mM solution of the peptide in DMSO 1:25 with assay buffer), and incubated in a fluorometer (Labsystems Fluoroskan) at 37° C. Hydrolysis of the substrate results in the release of free AMC, which was measured fluorometrically by using λ_{ex} =390 nm and λ_{em} =460 nm. The rate of hydrolysis in this system is linear for at least one hour. The hydrolysis rate of each sample is then normalized to relative fluorescent units per milligram of protein (RFU/mg).

[0417] To explore the *in vivo* activity of Salinosporamide A, male Swiss-Webster mice (5 per group, 20-25 g in weight) were treated with various concentrations of Salinosporamide A. Salinosporamide A was administered intravenously and given its $LogD^{7.4}$ value of 2.4, suggestive of oral availability, Salinosporamide A was also administered orally. Salinosporamide A dosing solutions were generated immediately prior to administration by dilution of Salinosporamide A stock solutions (100% DMSO) using 10% solutol yielding a final concentration of 2% DMSO. The vehicle control consisted of 2% DMSO in 10% solutol. One group of animals was not dosed with either vehicle or Salinosporamide A in order to establish a baseline for proteasome activity. Salinosporamide A or vehicle was administered at 10 mL/kg and ninety minutes after administration the animals were anesthetized and blood withdrawn by cardiac puncture. Packed whole blood cells were col-

lected by centrifugation, washed with PBS, and re-centrifuged. All samples were stored at -80° C. prior to the evaluation of the proteasome activity.

[0418] In order to be certain that the hydrolysis of the substrate observed in these experiments was due solely to the activity of the proteasome, dose response experiments on the extracts were performed using the highly specific proteasomal inhibitor Epoxomicin. PWBL lysates were diluted 1:40 in assay buffer, and 180 μ L were added to Costar 3904 plates. Epoxomicin (Calbiochem Cat. 324800) was serially diluted in DMSO to generate an eight point dose response curve, diluted 1:50 in assay buffer, and 10 μ L added to the diluted PWBL in triplicate. The samples were preincubated for 5 minutes at 37° C., and the reactions initiated with substrate as above. The dose response curves were analyzed in Prism, using a sigmoidal dose response with variable slope as a model.

[0419] FIG. 40 is a scatter plot displaying the normalized proteasome activity in PWBL's derived from the individual mice (5 mice per group). In each group, the horizontal bar represents the mean normalized activity. These data show that Salinosporamide A causes a profound decrease in proteasomal activity in PWBL, and that this inhibition is dose dependent. In addition, these data indicate that Salinosporamide A is active upon oral administration.

[0420] The specificity of the assay was shown by examining the effect of a known proteasome inhibitor, Epoxomicin, on hydrolysis of the peptide substrate. Epoxomicin is a peptide epoxide that has been shown to highly specific for the proteasome, with no inhibitory activity towards any other known protease (Meng et al., 1999). Lysates from a vehicle control and also from animals treated intravenously (i.v.) with 0.1 mg/kg Salinosporamide A were incubated with varying concentration of Epoxomicin, and IC_{50} values were determined. Palayoor et al., *Oncogene* 18:7389-94 (1999). As shown in FIG. 41, Epoxomicin caused a dose dependent inhibition in the hydrolysis of the proteasome substrate. The IC_{50} obtained in these experiments matches well with the 10 nM value observed using purified 20S proteasomes *in vitro* (not shown). These data also indicate that the remaining activity towards this substrate in these lysates prepared from animals treated with 0.1 mg/kg Salinosporamide A is due to the proteasome, and not some other protease. The residual activity seen in extracts treated with high doses of Epoxomicin is less than 2% of the total signal, indicating that over 98% of the activity observed with suc-LLVY-AMC as a substrate is due solely to the activity of the proteasomes present in the PWBL.

[0421] Comparison of intra-run variation in baseline activity and the ability of Salinosporamide A to inhibit proteasomal activity was also assessed. In FIG. 42, the results of separate assays run several weeks apart are shown. Qureshi, et al., *J. Immunol.* 171(3): 1515-25 (2003). For clarity, only the vehicle control and matching dose results are shown. While there was some variation in the proteasomal activity in PWBL derived from individual animals in the control groups, the overall mean was very similar between the two groups. The animals treated with Salinosporamide A (0.1 mg/kg i.v.) also show very similar residual activity and average inhibition. This suggests that results between assays can be compared with confidence.

Example 39

Inhibition of in vivo LPS-Induced TNF by Salinosporamide A

[0422] Studies suggest that the proteasome plays a role in the activation of many signaling molecules, including the transcription factor NF- κ B via proteolytic degradation of the inhibitor of NF- κ B (I κ B). LPS signaling through the TLR4 receptor activates NF- κ B and other transcriptional regulators resulting in the expression of a host of proinflammatory genes like TNF, IL-6, and IL-1 β . The continued expression of proinflammatory cytokines has been identified as a major factor in many diseases. Inhibitors of TNF and IL-1 β have shown efficacy in many inflammation models including the LPS murine model, as well as animal models of rheumatoid arthritis and inflammatory bowel disease. Recent studies have suggested that inhibition of the proteasome can prevent LPS-induced TNF secretion (Qureshi et al., 2003). These data suggest that Salinosporamide A, a novel potent proteasome inhibitor, may prevent TNF secretion in vivo in the high-dose LPS murine model.

[0423] To assess the ability of Salinosporamide A to inhibit in vivo LPS-induced plasma TNF levels in mice, in vivo studies were initiated at BolderBioPATH, Inc. in Boulder, Colo. The following methods outline the protocol design for these studies.

[0424] Male Swiss Webster mice (12/group weighing 20-25 g) were injected with LPS (2 mg/kg) by the i.p. route. Thirty minutes later, mice were injected i.v. (tail vein) with Salinosporamide A at 2.5 mg/kg after approximately 5 minutes under a heat lamp. Ninety minutes after LPS injection, the mice were anesthetized with Isoflurane and bled by cardiac puncture to obtain plasma. Remaining blood pellet was then resuspended in 500 μ L of PBS to wash away residual serum proteins and centrifuged again. Supernatant was removed and blood pellet frozen for analysis of proteasome inhibition in packed whole blood lysate.

TABLE 12

Group ID	Group	n	Time	
			0 min	+30 min
No injections/baseline	1	5		
Saline + solutol vehicle	2	5	saline	
Saline + solutol vehicles	3	5	saline	Solutol/DMSO
LPS Ip./Vehicle (-30 min)	4	12	LPS	
LPS Ip./Vehicle (+30 min)	5	12	LPS	Solutol/DMSO
saline/Salinosporamide A (-30 min) 0.25 mg/kg	6	12	saline	
saline/Salinosporamide A (+30 min) 0.25 mg/kg	7	12	saline	0.25 mg/kg
LPS/Salinosporamide A (-30 min) 0.25 mg/kg	8	12	LPS	
LPS/Salinosporamide A (+30 min) 0.25 mg/kg	9	12	LPS	0.25 mg/kg

[0425] Dosing solutions were prepared using a 10 mg/mL Salinosporamide A stock solution in 100% DMSO. A 10% solutol solution was prepared by diluting w/w with endotoxin-free water and a 1:160 dilution was made of the 10 mg/mL Salinosporamide A stock. Animals were dosed i.v. with 4 mL/kg. A vehicle control solution was also prepared by making the same 1:160 dilution with 100% DMSO into

10% solutol solution giving a final concentration of 9.375% solutol in water and 0.625% DMSO. Measurements of plasma TNF were performed using the Biosource mTNF Cytoset kit (Biosource Intl., Camarillo, Calif.; catalog #CMC3014) according to manufacturer's instructions. Samples were diluted 1:60 for the assay.

[0426] Data from two independent experiments with at least ten replicate animals per group indicated that treatment with 0.125 or 0.25 mg/kg Salinosporamide A decreased LPS-induced TNF secretion in vivo. A representative experiment is shown in FIG. 43. These data reveal that treatment of animals with 0.25 mg/kg Salinosporamide A thirty minutes after 2 mg/kg LPS injection resulted in significant reduction in serum TNF levels. Packed whole blood samples were also analyzed for ex vivo proteasome inhibition revealing 70 \pm 3% inhibition in animals treated with 0.125 mg/kg and 94 \pm 3% in animals treated with 0.25 mg/kg. No significant differences were seen in proteasome inhibition in animals treated with or without LPS. Salinosporamide A reduces LPS-induced plasma TNF levels by approximately 65% when administered at 0.125 or 0.25 mg/kg i.v. into mice 30 minutes post-LPS treatment.

Example 40

Potential in vitro Chemosensitizing Effects of Salinosporamide A

[0427] Chemotherapy agents such as CPT-11 (Irinotecan) can activate the transcription factor nuclear factor-kappa B (NF- κ B) in human colon cancer cell lines including LoVo cells, resulting in a decreased ability of these cells to undergo apoptosis. Cusack, et al., *Cancer Res* 61:3535 (2001). In unstimulated cells, NF- κ B resides in the cytoplasm in an inactive complex with the inhibitory protein I κ B (inhibitor of NF- κ B). Various stimuli can cause I κ B phosphorylation by I κ B kinase, followed by ubiquitination and degradation of I κ B by the proteasome. Following the degradation of I κ B, NF- κ B translocates to the nucleus and regulates gene expression, affecting many cellular processes, including upregulation of survival genes thereby inhibiting apoptosis.

[0428] The recently approved proteasome inhibitor, VelcadeTM (PS-341; Millennium Pharmaceuticals, Inc.), is directly toxic to cancer cells and can also enhance the cytotoxic activity of CPT-11 against LoVo cells in vitro and in a LoVo xenograft model by inhibiting proteasome induced degradation of I κ B. Adams, J., *Eur J Haematol* 70:265 (2003). In addition, VelcadeTM was found to inhibit the expression of proangiogenic chemokines/cytokines GRO- α and VEGF in squamous cell carcinoma, presumably through inhibition of the NF- κ B pathway. Sunwoo, et al., *Clin Cancer Res* 7:1419 (2001). The data indicate that proteasome inhibition may not only decrease tumor cell survival and growth, but also angiogenesis.

Example 41

Growth Inhibition of Colon, Prostate, Breast, Lung, Ovarian, Multiple Myeloma and Melanoma

[0429] Human colon adenocarcinoma (HT-29; HTB-38), prostate adenocarcinoma (PC-3; CRL-1435), breast adenocarcinoma (MDA-MB-231; HTB-26), non-small cell lung carcinoma (NCI-H292; CRL-1848), ovarian adenocarci-

noma (OVCAR-3; HTB-161), multiple myeloma (RPMI 8226; CCL-155), multiple myeloma (U266; TIB-196) and mouse melanoma (B16-F10; CRL-6475) cells were all purchased from ATCC and maintained in appropriate culture media. The cells were cultured in an incubator at 37° C. in 5% CO₂ and 95% humidified air.

[0430] For cell growth inhibition assays, HT-29, PC-3, MDA-MB-231, NCI-H292, OVCAR-3 and B16-F10 cells were seeded at 5×10^3 , 5×10^3 , 1×10^4 , 4×10^3 , 1×10^4 and 1.25×10^4 cells/well respectively in 90 μ l complete media into 96 well (Corning; 3904) black-walled, clear-bottom tissue culture plates and the plates were incubated overnight to allow cells to establish and enter log phase growth. RPMI

concentration at which 50% of the maximal observed growth inhibition is established) were determined using a standard sigmoidal dose response curve fitting algorithm (XLfit 3.0, ID Business Solutions Ltd). Where the maximum inhibition of cell growth was less than 50%, an EC₅₀ value was not determined.

[0432] The data in Table 13 summarize the growth inhibitory effects of Formule II-2, II-3, II-4, II-5A, II-5B, II-17, II-18 and II-19 against the human colorectal carcinoma, HT-29, human prostate carcinoma, PC-3, human breast adenocarcinoma, MDA-MB-231, human non-small cell lung carcinoma, NCI-H292, human ovarian carcinoma, OVCAR-3, human multiple myelomas, RPMI 8226 and U266 and murine melanoma B16-F10 cell lines.

TABLE 13

EC ₅₀ values of Formule II-2, II-3, II-4, II-5A, II-5B, II-17, II-18 and II-19 against various tumor cell lines								
	EC ₅₀ (nM)*							
Cell line	II-2	II-3	II-4	II-5A	II-5B	II-17	II-18	II-19
HT-29	129 ± 21	>20000	132 ± 36	85	1070	>20000	18 ± 7.8	13
PC-3	284 ± 110	>20000	204 ± 49	97	1330	>20000	35 ± 5.6	27
MDA-MB-231	121 ± 23	>20000	114 ± 4	66	1040	5900 ± 601	16 ± 2.8	17
NCI-H292	322	>20000	192	90	>20000	>20000	29	31
	395	>20000	213			>20000	41	
OVCAR-3	188	>20000	>6320	NT	NT	>20000	>2000	NT
	251		>6320			>20000	>2000	
RPMI 8226	49	>20000	57	36	326	6200	6.3	5.9
	45	>20000	51	29	328	3500	6.3	7.1
U266	39	>20000	39	10	118	1620	4.2	3.2
	32	>20000	34	9	111	1710	4.2	3.4
B16-F10	194	>20000	163	NT	NT	10500	19	NT
	180	>20000	175			10300	36	

*Where n = 3, mean \pm standard deviation is presented; NT = not tested

8226 and U266 cells were seeded at 2×10^4 and 2.5×10^4 cells/well respectively in 90 μ l complete media into 96 well plates on the day of the assay. 20 mM stock solutions of the compounds were prepared in 100% DMSO and stored at -80° C. The compounds were serially diluted and added in triplicate to the test wells. Concentrations ranging from 6.32 μ M to 632 μ M were tested for II-2 and II-4. II-3 and II-17 were tested at concentrations ranging from 20 μ M to 6.32 nM. Formula II-18 and II-19 were tested at concentrations ranging from 2 μ M to 200 μ M. Formula II-5A and Formula II-5B were tested at final concentrations ranging from 2 μ M to 632 μ M and 20 μ M to 6.32 nM respectively. The plates were returned to the incubator for 48 hours. The final concentration of DMSO was 0.25% in all samples.

[0431] Following 48 hours of drug exposure, 10 μ l of 0.2 mg/ml resazurin (obtained from Sigma-Aldrich Chemical Co.) in Mg²⁺, Ca²⁺ free phosphate buffered saline was added to each well and the plates were returned to the incubator for 3-6 hours. Since living cells metabolize Resazurin, the fluorescence of the reduction product of Resazurin was measured using a Fusion microplate fluorometer (Packard Bioscience) with λ_{ex} =535 nm and λ_{em} =590 nm filters. Resazurin dye in medium without cells was used to determine the background, which was subtracted from the data for all experimental wells. The data were normalized to the average fluorescence of the cells treated with media +0.25% DMSO (100% cell growth) and EC₅₀ values (the drug

[0433] The EC₅₀ values indicate that the Formule II-2, II-4 and II-18 were cytotoxic against the HT-29, PC-3, MDA-MB-231, NCI-H292, RPMI 8226, U266 and B16-F10 tumor cell lines. II-2 was also cytotoxic against the OVCAR-3 tumor cells. Formula II-17 was cytotoxic against MDA-MB-231, RPMI 8226, U266 and B16-F10 tumor cell lines. Formule II-5A, II-5B and II-19 were cytotoxic against HT-29, PC-3, MDA-MB-231, RPMI 8226 and U266 tumor cells. Formula II-5A and II-19 were also cytotoxic against NCI-H292 tumor cells.

[0434] The data in Table 15 summarize the growth inhibitory effects of Formule II-2, II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-16, II-17, II-18, II-19, IV-3C and Formula II-20 against the human multiple myeloma cell lines, RPMI 8226 and U266.

TABLE 15

Mean EC ₅₀ values of Formule II-2, II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-16, II-17, II-18, II-19, IV-3C and Formula II-20 against RPMI 8226 and U266 cells		
Compound	RPMI 8226 EC ₅₀ (nM)	U266 EC ₅₀ (nM)

Formula II-17	4800	1670
Formula II-16	7.0	4.1
Formula II-18	6.3	4.2
Formula II-2	47	36

TABLE 15-continued

Mean EC ₅₀ values of Formulae II-2, II-3, II-4, II-5A, II-5B, II-8C, II-13C, II-16, II-17, II-18, II-19, IV-3C and Formula II-20 against RPMI 8226 and U266 cells			
Compound	RPMI 8226 EC ₅₀ (nM)	U266 EC ₅₀ (nM)	
Formula II-3	>20000	>20000	
Formula II-4	54	36	
Formula II-5A	33	30	
Formula II-5B	327	115	
Formula II-8C	>20000	>20000	
Formula II-13C	>20000	>20000	
Formula II-19	6.5	3.3	
Formula IV-3C	>20000	8020	
Formula II-20*	10500	3810	

*n = 1

[0435] The EC₅₀ values indicate that Formulae II-2, II-4, II-5A, II-5B, II-16, II-17, II-18, II-19 and II-20 were cytotoxic against RPMI 8226 and U266 cells. Formula IV-3C was cytotoxic against U266 cells

Example 42

Growth Inhibition of MES-SA, MES-SA/Dx5, HL-60 and HL-60/MX2 Tumor Cell Lines

[0436] Human uterine sarcoma (MES-SA; CRL-1976), its multidrug resistant derivative (MES-SA/Dx5; CRL-1977), human acute promyelocytic leukemia cells (HL-60; CCL-240) and its multidrug resistant derivative (HL-60/MX2; CRL-2257) were purchased from ATCC and maintained in appropriate culture media. The cells were cultured in an incubator at 37° C. in 5% CO₂ and 95% humidified air.

[0437] For cell growth inhibition assays, MES-SA and MES-SA/Dx5 cells were both seeded at 3×10⁵ cells/well in 90 μ l complete media into 96 well (Corning; 3904) black-walled, clear-bottom tissue culture plates and the plates were incubated overnight to allow cells to establish and enter log phase growth. HL-60 and HL-60/MX2 cells were both seeded at 5×10⁴ cells/well in 90 μ l complete media into 96 well plates on the day of compound addition. 20 mM stock solutions of the compounds were prepared in 100% DMSO and stored at -80° C. The compounds were serially diluted and added in triplicate to the test wells. Concentrations ranging from 6.32 μ M to 2 nM were tested for II-2 and II-4. II-3 and II-17 were tested at concentrations ranging from 20 μ M to 6.32 nM. Compound II-18 was tested at concentrations ranging from 2 μ M to 632 pM. The plates were returned to the incubator for 48 hours. The final concentration of DMSO was 0.25% in all samples.

[0438] Following 48 hours of drug exposure, 10 μ l of 0.2 mg/ml resazurin (obtained from Sigma-Aldrich Chemical Co.) in Mg²⁺, Ca²⁺ free phosphate buffered saline was added to each well and the plates were returned to the incubator for 3-6 hours. Since living cells metabolize Resazurin, the fluorescence of the reduction product of Resazurin was measured using a Fusion microplate fluorometer (Packard Bioscience) with λ_{ex} =535 nm and λ_{em} =590 nm filters. Resazurin dye in medium without cells was used to determine the background, which was subtracted from the data for all experimental wells. The data were normalized to the average fluorescence of the cells treated with media +0.25%

DMSO (100% cell growth) and EC₅₀ values (the drug concentration at which 50% of the maximal observed growth inhibition is established) were determined using a standard sigmoidal dose response curve fitting algorithm (XLfit 3.0, ID Business Solutions Ltd). Where the maximum inhibition of cell growth was less than 50%, an EC₅₀ value was not determined.

[0439] The multidrug resistant MES-SA/Dx5 tumor cell line was derived from the human uterine sarcoma MES-SA tumor cell line and expresses elevated P-Glycoprotein (P-gp), an ATP dependent efflux pump. The data in Table 16 summarize the growth inhibitory effects of Formulae II-2, II-3, II-4, II-17 and II-18 against MES-SA and its multidrug resistant derivative MES-SA/Dx5. Paclitaxel, a known substrate of the P-gp pump was included as a control.

TABLE 16

EC ₅₀ values of Formulae II-2, II-3, II-4, II-17 and II-18 against MES-SA and MES-SA/Dx5 tumor cell lines			
Compound	EC ₅₀ (nM)		Fold change*
	MES-SA	MES-SA/Dx5	
II-2	193	220	1.0
	155	138	
II-3	>20000	>20000	NA
	>20000	>20000	
II-4	163	178	0.9
	140	93	
II-17	9230	9450	0.8
	12900	7530	
II-18	22	32	1.2
	17	14	
Paclitaxel	5.6	2930	798
	4.6	5210	

*Fold change = the ratio of EC₅₀ values (MES-SA/Dx5:MES-SA)

[0440] The EC₅₀ values indicate that II-2, II-4, II-17 and II-18 have cytotoxic activity against both MES-SA and MES-SA/Dx5 tumor cell lines. The multidrug resistant phenotype was confirmed by the observation that Paclitaxel was ~800 times less active against the resistant MES-SA/Dx5 cells.

[0441] HL-60/MX2 is a multidrug resistant tumor cell line derived from the human promyelocytic leukemia cell line, HL-60 and expresses reduced topoisomerase II activity. The data presented in Table 17 summarize the growth inhibitory effects of Formulae II-2, II-3, II-4, II-17 and II-18 against HL-60 and its multidrug resistant derivative HL-60/MX2. Mitoxantrone, the topoisomerase II targeting agent was included as a control.

TABLE 17

EC ₅₀ values of Formulae II-2, II-3, II-4, II-17 and II-18 against HL-60 and HL-60/MX2 tumor cell lines			
Compound	EC ₅₀ (nM)		Fold change*
	HL-60	HL-60/MX2	
II-2	237	142	0.7
	176	133	
II-3	>20000	>20000	NA
	>20000	>20000	

TABLE 17-continued

EC ₅₀ values of Formulae II-2, II-3, II-4, II-17 and II-18 against HIL-60 and HIL-60/MX2 tumor cell lines			
Compound	EC ₅₀ (nM)		Fold change*
	HIL-60	HIL-60/MX2	
II-4	143	103	0.8
	111	97	
II-17	>20000	>20000	NA
II-18	27	19	0.7
	23	18	
Mitoxantrone	42	1340	30.6
	40	1170	

*Fold change = the ratio of EC₅₀ values (HIL-60/MX2:HIL-60)

[0442] The EC₅₀ values indicate that II-2, II-4 and II-18 retained cytotoxic activity against both HIL-60 and HIL-60/MX2 tumor cell lines. The multidrug resistant phenotype was confirmed by the observation that Mitoxantrone was ~30 times less active against the resistant HIL-60/MX2 cells.

Example 43

Inhibition of NF-κB-Mediated Luciferase Activity: HEK293 NF-κB/Luciferase Reporter Cell Line

[0443] The HEK293 NF-κB/luciferase reporter cell line is a derivative of the human embryonic kidney cell line (ATCC; CRL-1573) and carries a luciferase reporter gene under the regulation of 5xNF-κB binding sites. The reporter cell line was routinely maintained in complete DMEM medium (DMEM plus 10% (v/v) Fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES and Penicillin/Streptomycin at 100 IU/ml and 100 µg/ml, respectively) supplemented with 250 µg/ml G418. When performing the luciferase assay, the DMEM basal medium was replaced with phenol-red free DMEM basal medium and the G418 was omitted. The cells were cultured in an incubator at 37° C. in 5% CO₂ and 95% humidified air.

[0444] For NF-κB-mediated luciferase assays, HEK293 NF-κB/luciferase cells were seeded at 1.5×10⁴ cells/well in 90 µl phenol-red free DMEM complete medium into Corning 3917 white opaque-bottom tissue culture plates. For Formula II-2, Formula II-4 and Formula II-5A, a 400 µM starting dilution was made in 100% DMSO and this dilution was used to generate a 8-point half log dilution series. This dilution series was further diluted 40x in appropriate culture medium and ten µl aliquots were added to the test wells in triplicate resulting in final test concentrations ranging from 1 µM to 320 pM. For Formula II-3 and Formula II-5B, a 8 mM starting dilution was made in 100% DMSO and the same procedure was followed as described above resulting in final test concentrations ranging from 20 µM to 6.3 nM. The plates were returned to the incubator for 1 hour. After 1 hr pretreatment, 10 µl of a 50 ng/ml TNF-α solution, prepared in the phenol-red free DMEM medium was added, and the plates were incubated for an additional 6 hr. The final concentration of DMSO was 0.25% in all samples.

[0445] At the end of the TNF-α stimulation, 100 µl of Steady Lite HTS Luciferase reagent (Packard Bioscience) was added to each well and the plates were left undisturbed for 10 min at room temperature before measuring the

luciferase activity. The relative luciferase units (RLU) were measured by using a Fusion microplate fluorometer (Packard Bioscience). The EC₅₀ values (the drug concentration at which 50% of the maximal relative luciferase unit inhibition is established) were calculated in Prism (GraphPad Software) using a sigmoidal dose response, variable slope model.

[0446] NF-κB regulates the expression of a large number of genes important in inflammation, apoptosis, tumorigenesis, and autoimmune diseases. Thus compounds capable of modulating or affecting NF-κB activity are useful in treating diseases related to inflammation, cancer, and autoimmune diseases, for example. In its inactive form, NF-κB complexes with IκB in the cytosol and upon stimulation, IκB is phosphorylated, ubiquitinated and subsequently degraded by the proteasome. The degradation of IκB leads to the activation of NF-κB and its translocation to the nucleus. The effects of Formula II-2, Formula II-3, Formula II-4, Formula II-5A and Formula II-5B on the activation of NF-κB was evaluated by assessing the NF-κB-mediated luciferase activity in HEK293 NF-κB/Luc cells upon TNF-α stimulation.

[0447] Results from a representative experiment evaluating Formula II-2, Formula II-3 and Formula II-4 (FIG. 44) revealed that pretreatment with Formula II-2 and Formula II-4 resulted in a dose-dependent decrease of luciferase activity in NF-κB/Luc 293 cells upon TNF-α stimulation. The calculated EC₅₀ to inhibit NF-κB inducible luciferase activity in this experiment was 73 nM for Formula II-2, while EC₅₀ value for Formula II-4 was 67 nM. Similar data were observed in a replicate experiment.

[0448] Results from a representative experiment evaluating Formula II-5A and Formula II-5B are shown in FIG. 45 and illustrate that Formula II-5A and Formula II-5B inhibit NF-κB inducible luciferase activity with EC₅₀ values of 30 nM and 261 nM respectively. Similar data were observed in a replicate experiment.

Example 44

In vitro Inhibition of Proteasome Activity by Formula II-2, Formula II-3, Formula II-4, Formula II-5A and Formula II-5B

[0449] Formula II-2, Formula II-3, Formula II-4, Formula II-5A and Formula II-5B were prepared as 20 mM stock solution in DMSO and stored in small aliquots at -80° C. Purified rabbit muscle 20S proteasome was obtained from CalBiochem. To enhance the chymotrypsin-like activity of the proteasome, the assay buffer (20 mM HEPES, pH7.3, 0.5 mM EDTA, and 0.05% Triton X100) was supplemented with SDS resulting in a final SDS concentration of 0.035%. The substrate used was sucLLVY-AMC, a fluorogenic peptide substrate specifically cleaved by the chymotrypsin-like activity of the proteasome. Assays were performed at a proteasome concentration of 1 µg/ml in a final volume of 200 µl in 96-well Costar microtiter plates. Formula II-2 and Formula II-4 were tested as eight-point dose response curves with final concentrations ranging from 500 nM to 0.16 nM, while Formula II-3 was tested with final concentrations ranging from 10 µM to 3.2 nM. Formula II-5A and Formula II-5B were tested with final concentrations ranging from 1 µM to 0.32 nM. The samples were incubated at 37° C. for five minutes in a temperature controlled plate reader. During

the preincubation step, the substrate was diluted 25-fold in assay buffer supplemented with 0.035% SDS. After the preincubation period, the reactions were initiated by the addition of 10 μ l of the diluted substrate and the plates were returned to the plate reader. The final concentration of substrate in the reactions was 20 μ M. All data were collected every five minutes for more than 1.5 hour and plotted as the mean of triplicate data points. The EC_{50} values (the drug concentration at which 50% of the maximal relative fluorescence unit is inhibited) were calculated by Prism (Graph-Pad Software) using a sigmoidal dose-response, variable slope model.

[0450] Results from a representative experiment evaluating Formula II-2, Formula II-3 and Formula II-4 are shown in FIG. 46 and illustrate that Formula II-2 and Formula II-4 inhibit the chymotrypsin-like activity of the proteasome with EC_{50} values of 18.5 nM and 15 nM respectively. Formula II-3 is active in this assay with an EC_{50} value of 890 nM. Similar results were obtained from an independent experiment.

[0451] Results from a representative experiment evaluating Formula II-5A and Formula II-5B are shown in FIG. 47 and illustrate that Formula II-5A and Formula II-5B inhibit the chymotrypsin-like activity of the proteasome with EC_{50} values of 6 nM and 88 nM respectively. Similar results were obtained in an independent experiment.

Example 45

Inhibition of Anthrax Lethal Toxin

[0452] Anthrax toxin is responsible for the symptoms associated with anthrax. In this disease, *B. anthracis* spores are inhaled and lodge in the lungs where they are ingested by macrophages. Within the macrophage, spores germinate, replicate, resulting in killing of the cell. Before killing occurs, however, infected macrophages migrate to the lymph nodes where, upon death, they release their contents, allowing the organism to enter the bloodstream, further replicate, and secrete lethal toxins.

[0453] Two proteins called protective antigen (PA 83 kDa) and lethal factor (LF, 90 kDa), play a key role in the pathogenesis of anthrax. These proteins are collectively known as lethal toxin (LeTx). When combined, PA and LF cause death when injected intravenously in animals. Lethal toxin is also active in a few cell culture lines of macrophages causing cell death within a few hours. LeTx can induce both necrosis and apoptosis in mouse macrophage-like RAW264.7 cells upon in vitro treatment.

[0454] In vitro Cell-Based Assay for Inhibitors of Lethal Toxin-Mediated Cytotoxicity

[0455] RAW264.7 cells (obtained from the American Type Culture Collection) were adapted to and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% Penicillin/Streptomycin (complete medium) at 37° C. in a humidified 5% CO₂ incubator. For the assay, cells were plated overnight in complete medium at a concentration of 50,000 cells/well in a 96-well plate. Media was removed the following day and replaced with serum-free complete medium with or without varying concentrations of Formulae II-2, II-3, II-4, II-5A, II-5B, II-13C, II-17, II-18 and IV-3C starting at 330 nM and

diluting at 1/2 log intervals for an 8-point dose-response. After a 45 minute preincubation, 1 μ g/ml LF and 1 μ g/ml PA alone or in combination (LF:PA, also termed lethal toxin (LeTx)) were added to cells. Recombinant LF and PA were obtained from List Biological Laboratories. Additional plates with no LeTx added were included as a control. Cells were then incubated for six hours followed by addition of 0.02 mg/ml resazurin dye (Molecular Probes, Eugene, Ore.) prepared in Mg++, Ca++ free PBS (Mediatech, Herndon, Va.). Plates were then incubated an additional 1.5 hours prior to the assessment of cell viability. Since resazurin is metabolized by living cells, cytotoxicity or cell viability can be assessed by measuring fluorescence using 530 excitation and 590 emission filters. Data are expressed as the percent viability as compared to a DMSO alone control (high) and the LeTx alone control (low) using the following equation: Percent viability = 100 * ((Measured OD-low control)/(high control-low control)).

[0456] Inhibition of Anthrax Lethal Toxin-mediated Cytotoxicity in RAW 264.7 Cells

[0457] Data in FIG. 48 summarize the effects of Formula II-2, Formula II-3 and Formula II-4 against LeTx-mediated cytotoxicity of the RAW 264.7 murine macrophage-like cell line. Treatment of RAW 264.7 cells with Formula II-2 and Formula II-4 resulted in an increase in the viability of LeTx treated cells with EC_{50} values of 14 nM (FIG. 48). The EC_{50} values for Formula II-3 for LeTx protection was not determined at the concentrations tested (EC_{50} > 330 nM, the maximum concentration evaluated). Data in Table 18 show the effects of Formulae II-5A, II-5B, II-13C, II-17, II-18 and IV-3C against LeTx-mediated cytotoxicity of the RAW 264.7 murine macrophage-like cell line. Treatment of RAW 264.7 cells with Formula II-5A and II-18 showed an increase in the viability of LeTx treated RAW 264.7 cells with EC_{50} values of 3 nM and 4 nM respectively. Treatment with Formula II-17 and Formula II-5B resulted in an increase in the viability of LeTx treated cells with EC_{50} values of 42 nM and 45 nM respectively. The EC_{50} values for Formulae II-13C and IV-3C for LeTx protection could not be determined at the concentrations tested (EC_{50} > 330 nM, the maximum concentration evaluated).

TABLE 18

EC ₅₀ values for inhibition of RAW 264.7 cell cytotoxicity mediated by anthrax lethal toxin	
Compound	EC ₅₀ (nM)
Formula II-17	42
Formula II-18	4
Formula II-5A	3
Formula II-5B	45
Formula II-13C	>330 nM
Formula IV-3C	>330 nM

Example 46

Formulation to be Administered Orally or the Like

[0458] A mixture obtained by thoroughly blending 1 g of a compound obtained and purified by the method of the embodiment, 98 g of lactose and 1 g of hydroxypropyl cellulose is formed into granules by any conventional method. The granules are thoroughly dried and sifted to

obtain a granule preparation suitable for packaging in bottles or by heat sealing. The resultant granule preparations are orally administered at between approximately 100 ml/day to approximately 1000 ml/day, depending on the symptoms, as deemed appropriate by those of ordinary skill in the art of treating cancerous tumors in humans.

[0459] The examples described above are set forth solely to assist in the understanding of the embodiments. Thus, those skilled in the art will appreciate that the methods may provide derivatives of compounds.

[0460] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and procedures described herein are presently representative of preferred embodiments and are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention.

[0461] It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the embodiments disclosed herein without departing from the scope and spirit of the invention.

[0462] All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

[0463] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions indicates the exclusion of equivalents of the features shown and described or portions thereof. It is recognized that various modifications are possible within the scope of the invention. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be falling within the scope of the invention, which is limited only by the following claims.

What is claimed is:

1. A method of treating a neoplastic disease in an animal, the method comprising:

administering to the animal, a therapeutically effective amount of a compound of a formula selected from Formulae I-V, and pharmaceutically acceptable salts and pro-drug esters thereof;

2. The method of claim 1, wherein the neoplastic disease is cancer.

3. The method of claim 2, wherein the cancer is selected from the group consisting of breast cancer, sarcoma, leukemia, ovarian cancer, ureteral cancer, bladder cancer, prostate cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, multiple myeloma, pancreatic cancer,

liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, and brain or central nervous system (CNS) cancer.

4. The method of claim 3, wherein the cancer is a multiple myeloma, a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma or a melanoma.

5. The method of claim 2, wherein the cancer is a drug resistant cancer.

6. The method of claim 5, wherein the drug-resistant cancer displays at least one of the following: elevated levels of the P-glycoprotein efflux pump, increased expression of the multidrug-resistance associated protein 1 encoded by MRP1, reduced drug uptake, alteration of the drug's target or increasing repair of drug-induced DNA damage, alteration of the apoptotic pathway or the activation of cytochrome P450 enzymes.

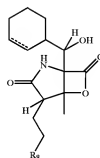
7. The method of claim 5, wherein the drug resistant cancer is a sarcoma or a leukemia.

8. The method of claim 1, wherein the animal is a mammal.

9. The method of claim 1, wherein the animal is a human.

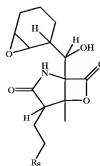
10. The method of claim 1, wherein the animal is a rodent.

11. The method of claim 1, wherein the the compound is:



wherein R_8 is selected from the group consisting of H, F, Cl, Br and I.

12. The method of claim 1, wherein the compound is:



wherein R_8 is selected from the group consisting of H, F, Cl, Br, and I.

13. The method of claim 1, further comprising the steps of:

identifying a subject that would benefit from administration of an anticancer agent;

performing the method on the subject.

14. A pharmaceutical composition comprising a compound of a formula selected from Formulae I-V, and pharmaceutically acceptable salts and pro-drug esters thereof.

15. The pharmaceutical composition of claim 14, further comprising an anti-microbial agent.

16. A method of inhibiting the growth of a cancer cell, comprising contacting a cancer cell with a compound of a formula selected from Formulae I-V, and pharmaceutically acceptable salts and pro-drug esters thereof.

17. The method of claim 16, wherein the cancer cell is a multiple myeloma, a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma and a melanoma.

18. A method of inhibiting proteasome activity comprising the step contacting a cell with a compound of a formula selected from Formulae I-V, and pharmaceutically acceptable salts and pro-drug esters thereof.

19. A method of inhibiting NF- κ B activation comprising the step contacting a cell with a compound of a formula selected from Formulae I-V, and pharmaceutically acceptable salts and pro-drug esters thereof.

20. A method for treating an inflammatory condition, comprising administering an effective amount of a compound of a formula selected from Formulae I-V to a patient in need thereof.

21. The method of claim 20, wherein the inflammatory condition is selected from the group consisting of rheumatoid arthritis, asthma, multiple sclerosis, psoriasis, stroke, and myocardial infarction.

22. A method for treating a microbial illness comprising administering an effective amount of a compound of a formula selected from Formulae I-V to a patient in need thereof.

23. The method of claim 22, wherein the microbial illness is caused by a microbe selected from the group consisting of *B. anthracis*, *Plasmodium*, *Leishmania*, and *Trypanosoma*.

* * * * *



US 20090036390A1

(19) **United States**(12) **Patent Application Publication****ANDERSON et al.**(10) **Pub. No.: US 2009/0036390 A1**(43) **Pub. Date: Feb. 5, 2009**(54) **COMPOSITIONS AND METHODS FOR
TREATING NEOPLASTIC DISEASES**(75) Inventors: **Kenneth C. ANDERSON,**
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INSTITUTE, Boston, MA (US)(21) Appl. No.: **12/183,007**(22) Filed: **Jul. 30, 2008****Related U.S. Application Data**(63) Continuation of application No. 11/293,354, filed on
Dec. 2, 2005, now abandoned.(60) Provisional application No. 60/633,161, filed on Dec.
3, 2004.**Publication Classification**(51) **Int. Cl.****A61K 31/407** (2006.01)**A61P 35/00** (2006.01)**A61K 31/704** (2006.01)**A61K 31/573** (2006.01)**A61K 31/454** (2006.01)**A61K 31/69** (2006.01)(52) **U.S. Cl. 514/34; 514/421; 514/171; 514/323;**
514/64(57) **ABSTRACT**Disclosed herein are compositions and methods for treating
neoplastic diseases. Included are compositions and methods
that are effective against multiple myeloma cells resistant to
conventional and bortezomib treatment. Furthermore, com-
bination treatment with two different proteasome inhibitors is
shown to be synergistic for treating multiple myeloma.

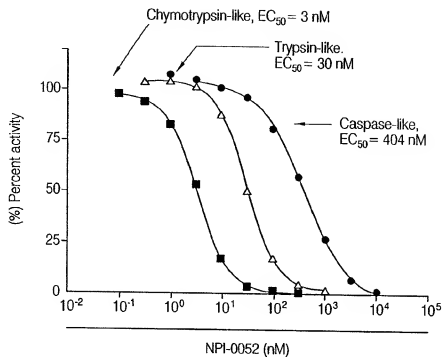


FIG. 1

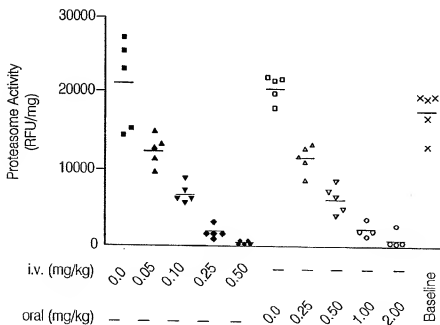


FIG. 2

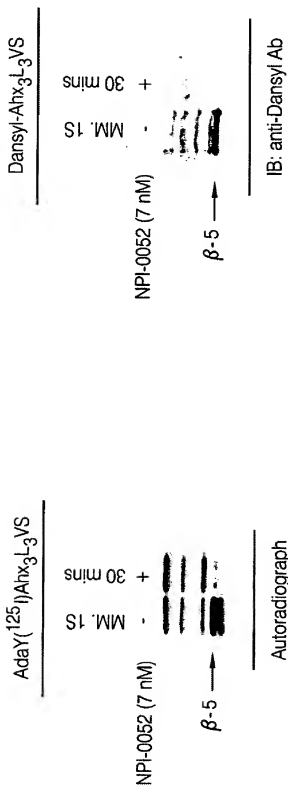


FIG. 3

FIG. 4

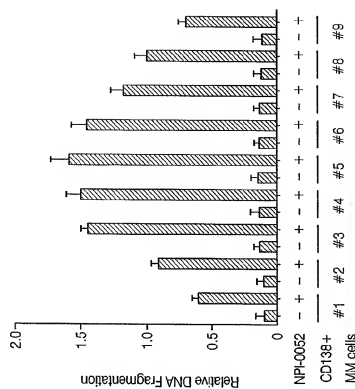


FIG. 5B

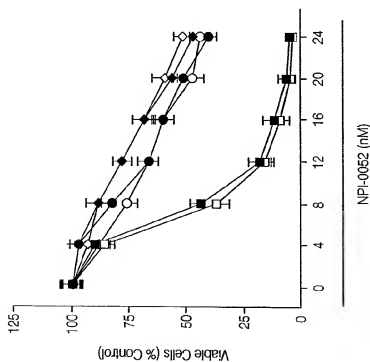
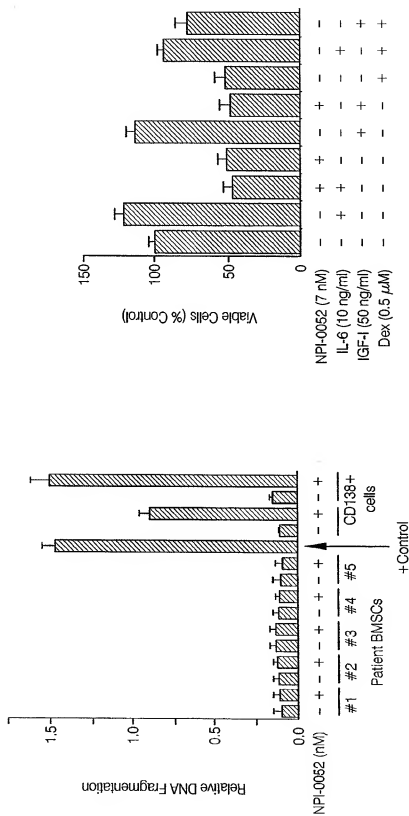


FIG. 5A



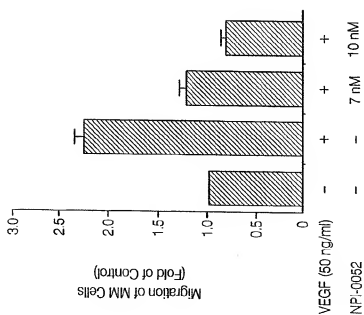


FIG. 8

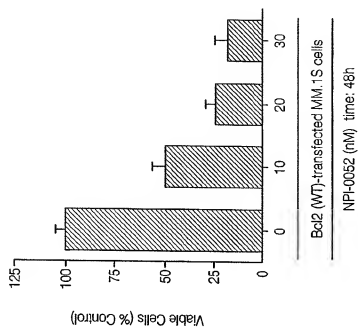


FIG. 9

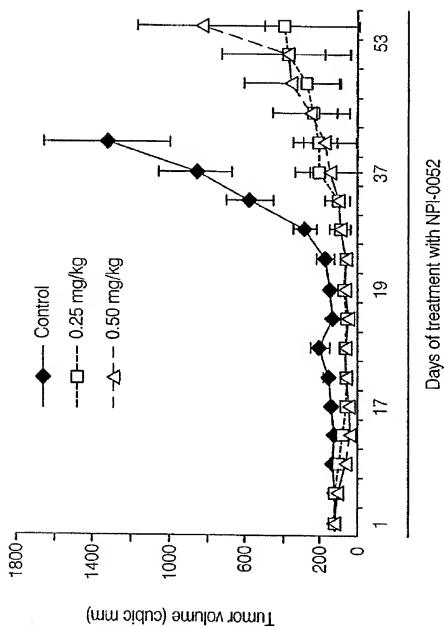


FIG. 10A

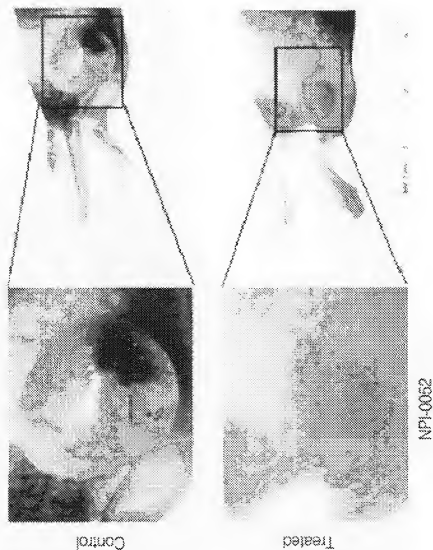


FIG. 10B

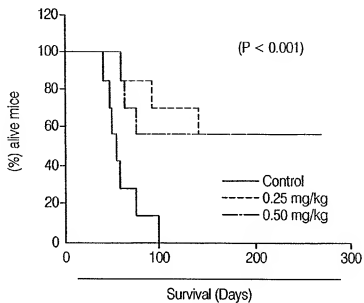


FIG. 10C

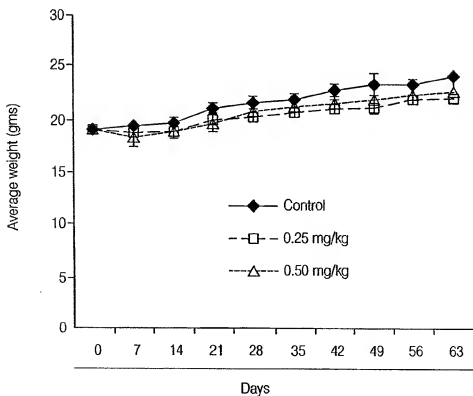


FIG. 10D

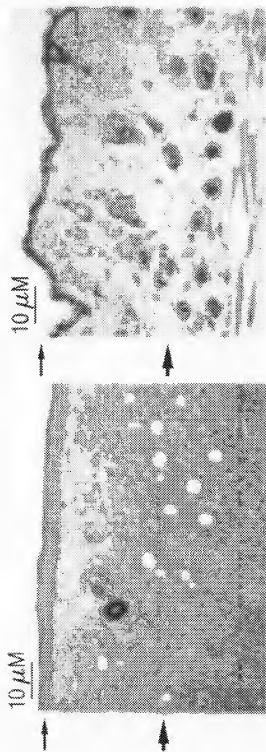


FIG. 10E

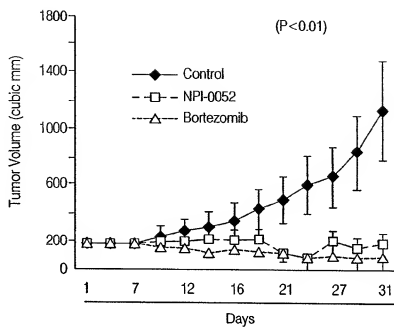


FIG. 10F

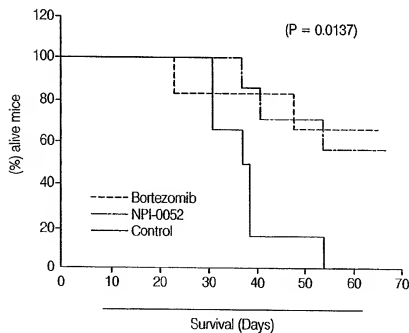


FIG. 10G

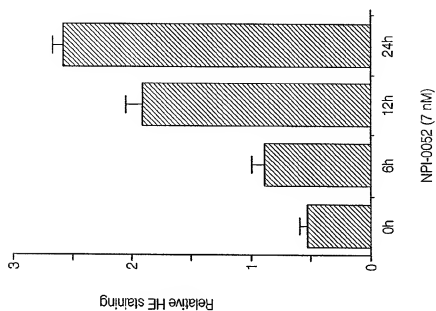


FIG. 11B

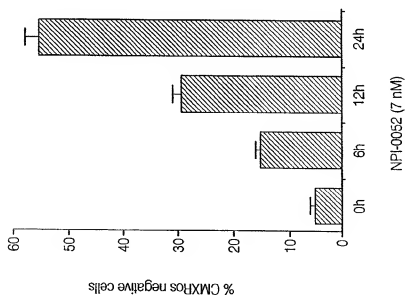


FIG. 11A

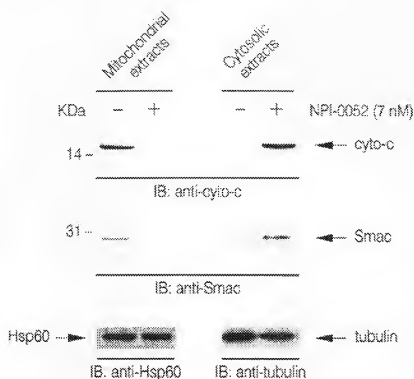


FIG. 11C

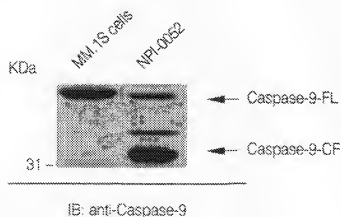


FIG. 11D

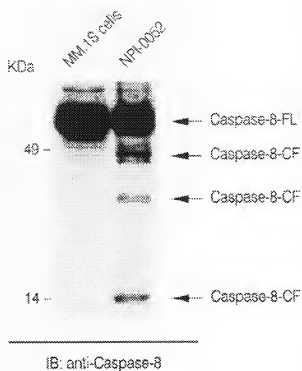


FIG. 11E

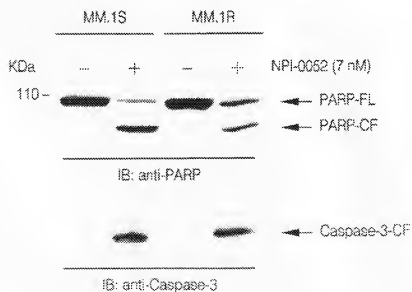


FIG. 11F

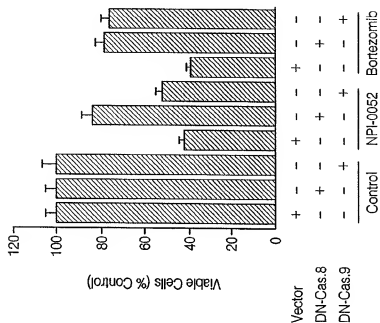


FIG. 12B

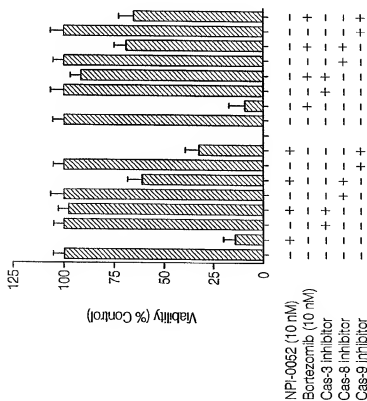


FIG. 12A

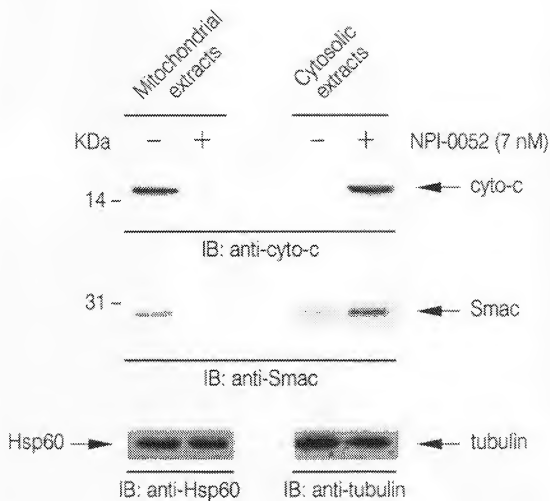


FIG. 12C

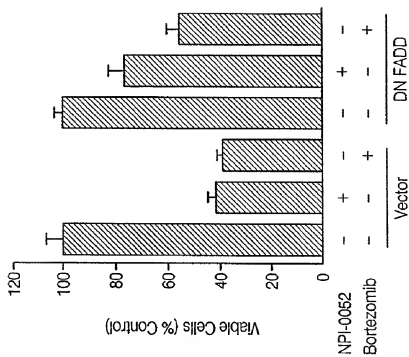
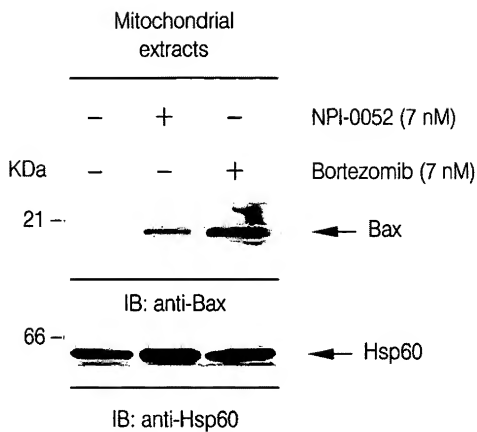


FIG. 12D



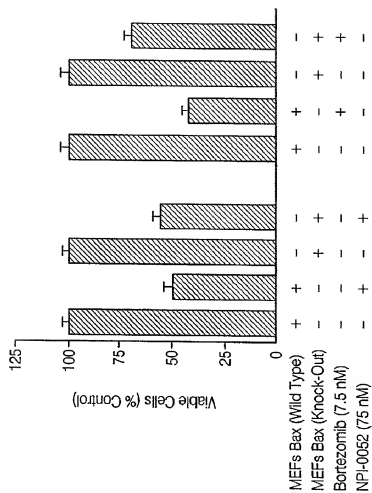


FIG. 12F

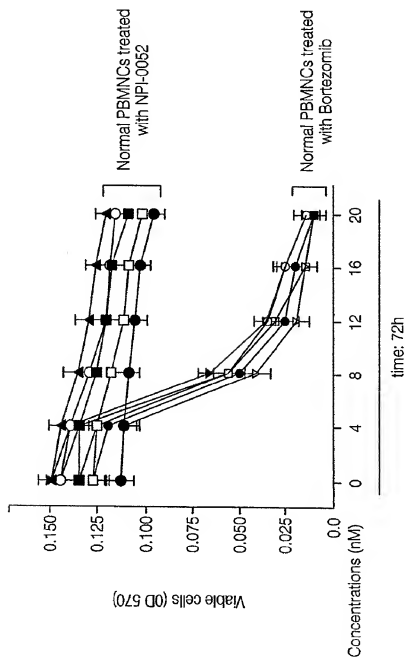


FIG. 13

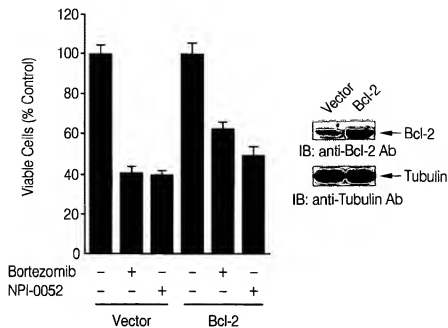


FIG. 14A

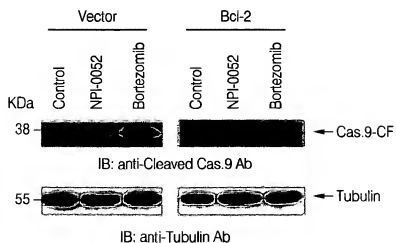


FIG. 14B

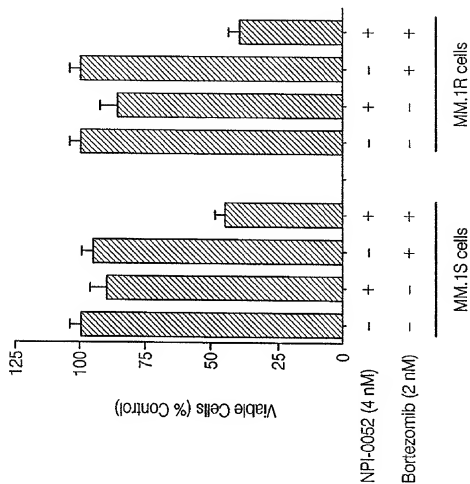


FIG. 15

COMPOSITIONS AND METHODS FOR TREATING NEOPLASTIC DISEASES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. application Ser. No. 11/293,354, filed Dec. 2, 2005, which claims the benefit of U.S. Provisional Application No. 60/633,161, filed Dec. 3, 2004, both of which are incorporated herein by reference in their entirety.

STATEMENT OF GOVERNMENT INTEREST

[0002] The work described herein was partially funded by NIH grants 50947, CA 78373, SPORE P50 CA100707-01, and P01 CA078378-06 and the U.S. government may have certain rights with regard to the present invention.

BACKGROUND OF THE INVENTION

[0003] 1. Field of the Invention

[0004] The present invention relates to the fields of chemistry and medicine. More particularly, the present invention relates to the treatment of neoplastic diseases, such as cancer.

[0005] 2. Description of the Related Art

[0006] Cancer is a leading cause of death in the United States. Despite significant efforts to find new approaches for treating cancer, the primary treatment options remain surgery, chemotherapy and radiation therapy, either alone or in combination. Surgery and radiation therapy, however, are generally useful only for fairly defined types of cancer, and are of limited use for treating patients with disseminated disease. Chemotherapy is the method that is generally useful in treating patients with metastatic cancer or diffuse cancers such as leukemias. Although chemotherapy can provide a therapeutic benefit, it often fails to result in cure of the disease due to the patient's cancer cells becoming resistant to the chemotherapeutic agent.

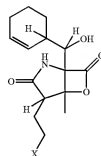
[0007] Therefore, a need exists for additional chemotherapeutics to treat cancer. A continuing effort is being made by individual investigators, academia and companies to identify new, potentially useful chemotherapeutic and anti-microbial agents.

[0008] The successful development of Bortezomib/PS-341 therapy for treatment of relapsed/refractory multiple myeloma (MM) has established proteasome inhibition as an effective therapeutic strategy. The dipeptide boronic acid analogue Bortezomib is a potent, highly selective, and reversible proteasome inhibitor which targets the 26S proteasome complex and inhibits its function. The 26S proteasome is an adenosine triphosphate (ATP)-dependent multicatalytic protease mediating intracellular protein degradation. Proteasomal degradation of misfolded or damaged proteins proceeds by recognition of polyubiquitinated proteins by the 19S regulatory subunit of the 26S protease, and subsequent hydrolysis to small polypeptides. Bortezomib primarily inhibits chymotrypsin, without altering trypsin or caspase-like, proteasome activity. Besides inhibiting NF- κ B, Bortezomib has pleiotropic effects on MM biology by targeting: 1) cell-cycle regulatory proteins; 2) UPR pathway via modulating transcriptional activity of plasma cell differentiation factor X-box binding protein-1 (XBP-1); 3) p53-mediated apoptosis/MDM2; 4) DNA repair mechanisms; 5) classical stress-response pathways via both intrinsic (caspase-9 mediated) and extrinsic (caspase-8 mediated) cell death cascades. Specifically, Bort-

ezomib activates JNK, which triggers mitochondrial apoptotic signaling: release of cytochrome-c (cyto-c) and second mitochondrial activator of caspases (Smac) from mitochondria to cytosol, followed by activation of caspase-9 and caspase-3. However, both intrinsic and acquired resistance has already been observed, and there are no therapies to overcome Bortezomib resistance at present.

SUMMARY OF THE INVENTION

[0009] One aspect of the present invention is a method of treating a neoplastic disease, comprising administering to a patient afflicted with the neoplastic disease a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:



wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine, and wherein the neoplastic disease is susceptible to resistance to at least one other chemotherapeutic agent.

[0010] Another aspect of the present invention is a method of treating a neoplastic disease, comprising administering to a patient afflicted with the neoplastic disease a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine, in combination with at least one additional chemotherapeutic agent.

[0011] Another aspect of the present invention is a pharmaceutical composition, comprising a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine, and at least one additional chemotherapeutic agent.

[0012] Another aspect of the present invention is a method of treating a neoplastic disease, comprising administering to a patient afflicted with the neoplastic disease a synergistic combination of at least two proteasome inhibitors.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] FIG. 1 illustrates inhibition of chymotrypsin-like, caspase-like, and trypsin-like proteasome activities in human erythrocytes-derived 20S proteasome by NPI-0052.

[0014] FIG. 2 illustrates the in vivo chymotrypsin-like activity of NPI-0052 in mice.

[0015] FIG. 3 depicts the autoradiograph obtained after treating MM.1S multiple myeloma (MM) cells with NPI-0052 (7 nM) and incubating protein extracts with Ada Y⁽¹²⁵⁾I Ahx₁L₃ VS at 37° C.

[0016] FIG. 4 depicts immunoblots obtained after treating MM.1S cells with NPI-0052 and then incubating with Dantyl-Ahx31.3VS.

[0017] FIG. 5A illustrates cell viability of various multiple myeloma cell lines treated with indicated doses of NPI-0052 for 24 h.

[0018] FIG. 5B illustrates DNA fragmentation assays of apoptosis after treatment with NPI-0052 of MM cells obtained from patients.

[0019] FIG. 6 illustrates DNA fragmentation assays of apoptosis after treatment with NPI-0052 of bone marrow stromal cells obtained from patients.

[0020] FIG. 7 illustrates MTT assay of MM.1S cell viability after treatment with NPI-0052 or Dex in the presence or absence of IL-6 or IGF-1.

[0021] FIG. 8 illustrates the effect of NPI-0052 on VEGF-induced migration of MM.1S cells.

[0022] FIG. 9 illustrates the effect of NPI-0052 on Bcl2-overexpressing MM.1S cell viability.

[0023] FIGS. 10A and 10B depict the effect of NPI-0052 on tumor growth when administered orally to mice.

[0024] FIG. 10C illustrates the effect of NPI-0052 on survival when administered orally to mice.

[0025] FIG. 10D illustrates the effect of NPI-0052 on body weight when administered orally to mice.

[0026] FIG. 10E illustrates tissue sections of inoculation sites from NPI-0052-treated and control-treated mice.

[0027] FIG. 10F compares the effect of NPI-0052 and Bortezomib on tumor growth when administered i.v. to mice.

[0028] FIG. 10G compares the effect of NPI-0052 and Bortezomib on survival when administered i.v. to mice.

[0029] FIG. 11A illustrates the effect of NPI-0052 on mitochondrial membrane potential in MM.1S cells incubated with CMXRos.

[0030] FIG. 11B illustrates the effect of NPI-0052 on superoxide generation in MM.1S cells stained with membrane permeable dye dihydroethidium (HE).

[0031] FIG. 11C depicts immunoblots of mitochondrial and cytosolic protein fractions obtained from MM.1S cells treated with NPI-0052.

[0032] FIG. 11D depicts immunoblots of cytosolic proteins obtained from MM.1S cells treated with NPI-0052 and analyzed with anti-caspase-9 Abs.

[0033] FIG. 11E depicts immunoblots of cytosolic proteins obtained from MM.1S cells treated with NPI-0052 and analyzed with anti-caspase-8 Abs.

[0034] FIG. 11F depicts immunoblots of MM.1S or MM.1R MM cells treated with NPI-0052 and assessed for apoptosis by both PARP and caspase-3 cleavage assays.

[0035] FIG. 12A illustrates MM.1S cell viability after treatment with NPI-0052 or Bortezomib in the presence or absence of caspase-3, caspase-8, or caspase-9 inhibitor.

[0036] FIG. 12B illustrates MM.1S cell viability for cells transfected with vector alone, DN-caspase-8, and DN-caspase-9 after treatment with NPI-0052 or Bortezomib.

[0037] FIG. 12C depicts immunoblots of cytosolic extracts from DN-caspase-8 and DN-caspase-9 transfected MM.1S cells treated with dexamethasone or anti-Fas MoAb.

[0038] FIG. 12D illustrates MM.1S cell viability for vector or DN-FADD transfected cells after treatment with NPI-0052 or Bortezomib.

[0039] FIG. 12E depicts immunoblots of mitochondrial protein extracts from MM.1S MM cells treated with indicated

concentration of either NPI-0052 or Bortezomib and analyzed with anti-Bax or anti-Hsp60 Abs.

[0040] FIG. 12F illustrates cell viability of mouse embryonic fibroblasts (MEFs) cells with either wild-type or deleted Bax (knock-out) treated with indicated concentrations of NPI-0052 or Bortezomib.

[0041] FIG. 13 illustrates viability of normal lymphocytes from five healthy donors treated with indicated concentrations of NPI-0052 or Bortezomib.

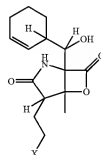
[0042] FIG. 14A illustrates MM.1S cell viability for cells transfected with vector alone or Bcl-2 after treatment with NPI-0052 or Bortezomib.

[0043] FIG. 14B depicts immunoblots of cytosolic extracts from vector- or Bcl-2-transfected MM.1S cells treated with NPI-0052 or Bortezomib.

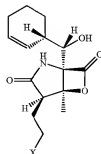
[0044] FIG. 15 illustrates cell viability of MM.1S and MM.1R MM cells treated with indicated concentration of NPI-0052, Bortezomib, or NPI-0052+Bortezomib.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0045] In one embodiment, a compound according to formula (I) is provided for use as described herein:



where X may be fluorine, chlorine, bromine or iodine. In one embodiment, X is chlorine. In one embodiment, the compound of formula (I) has stereochemistry according to formula (II):



The compound of formula (II) where X=Cl is also referred to herein as NPI-0052. Compounds according to formulae (I) or (II) may be derived from fermentation of *Salinispora*, a marine gram-positive actinomycete.

[0046] In some embodiments, prodrugs, metabolites, stereoisomers, and pharmaceutically acceptable salts of the compounds disclosed herein are provided for use as described herein.

[0047] A "prodrug" refers to an agent that is converted into the parent drug in vivo. Prodrugs are often useful because, in some situations, they may be easier to administer than the parent drug. They may, for instance, be bioavailable by oral administration whereas the parent is not. The prodrug may also have improved solubility in pharmaceutical compositions over the parent drug. An example, without limitation, of a prodrug would be a compound which is administered as an ester (the "prodrug") to facilitate transmittal across a cell membrane where water solubility is detrimental to mobility but which then is metabolically hydrolyzed to the carboxylic acid, the active entity, once inside the cell where water-solubility is beneficial. A further example of a prodrug might be a short peptide (polypeptide) bonded to an acid group where the peptide is metabolized to reveal the active moiety. Conventional procedures for the selection and preparation of suitable prodrug derivatives are described, for example, in *Design of Prodrugs*, (ed. H. Bundgaard, Elsevier, 1985), which is hereby incorporated herein by reference in its entirety.

[0048] The term "pro-drug ester" refers to derivatives of the compounds disclosed herein formed by the addition of any of several ester-forming groups that are hydrolyzed under physiological conditions. Examples of pro-drug ester groups include pivaloyloxymethyl, acetoxymethyl, phthalidyl, indanyl and methoxymethyl, as well as other such groups known in the art, including a (5-R-2-oxo-1,3-dioxolen-4-yl)methyl group. Other examples of pro-drug ester groups can be found in, for example, T. Higuchi and V. Stella, in "Pro-drugs as Novel Delivery Systems", Vol. 14, A.C.S. Symposium Series, American Chemical Society (1975); and "Bioversible Carriers in Drug Design: Theory and Application", edited by E. B. Roche, Pergamon Press: New York, 14-21 (1987) (providing examples of esters useful as prodrugs for compounds containing carboxyl groups). Each of the above-mentioned references is herein incorporated by reference in their entirety.

[0049] Metabolites of the compounds disclosed herein include active species that are produced upon introduction of the compounds into the biological milieu.

[0050] Where the compounds disclosed herein have at least one chiral center, they may exist as a racemate or as enantiomers. It should be noted that all such isomers and mixtures thereof are included in the scope of the present invention. Furthermore, some of the crystalline forms for the compounds of disclosed herein may exist as polymorphs. Such polymorphs are included in one embodiment of the present invention. In addition, some of the compounds of the present invention may form solvates with water (i.e., hydrates) or common organic solvents. Such solvates are included in one embodiment of the present invention.

[0051] The term "pharmaceutically acceptable salt" refers to a salt of a compound that does not cause significant irritation to an organism to which it is administered and does not abrogate the biological activity and properties of the compound. In some embodiments, the salt is an acid addition salt of the compound. Pharmaceutical salts can be obtained by reacting a compound with inorganic acids such as hydrohalic acid (e.g., hydrochloric acid or hydrobromic acid), sulfuric acid, nitric acid, phosphoric acid and the like. Pharmaceutical

salts can also be obtained by reacting a compound with an organic acid such as aliphatic or aromatic carboxylic or sulfonic acids, for example acetic, succinic, lactic, malic, tartaric, citric, ascorbic, nicotinic, methanesulfonic, ethanesulfonic, p-toluenesulfonic, salicylic or naphthalenesulfonic acid. Pharmaceutical salts can also be obtained by reacting a compound with a base to form a salt such as an ammonium salt, an alkali metal salt, such as a sodium or a potassium salt, an alkaline earth metal salt, such as a calcium or a magnesium salt, a salt of organic bases such as dicyclohexylamine, N-methyl-D-glucamine, tri(hydroxymethyl)methylamine, C₁-C₇ alkylamine, cyclohexylamine, triethanolamine, ethylenediamine, and salts with amino acids such as arginine, lysine, and the like.

[0052] If the manufacture of pharmaceutical formulations involves intimate mixing of the pharmaceutical excipients and the active ingredient in its salt form, then it may be desirable to use pharmaceutical excipients which are non-basic, that is, either acidic or neutral excipients.

[0053] In various embodiments, the compounds disclosed herein can be used alone, in combination with other compounds disclosed herein, or in combination with one or more other agents active in the therapeutic areas described herein.

[0054] The term "halogen atom," as used herein, means any one of the radio-stable atoms of column 7 of the Periodic Table of the Elements, e.g., fluorine, chlorine, bromine, or iodine, with fluorine and chlorine being preferred.

[0055] The term "ester" refers to a chemical moiety with formula $-(R)_n-COOR'$, where R and R' are independently selected from the group consisting of alkyl, cycloalkyl, aryl, heteroaryl (bonded through a ring carbon) and heterocyclic (bonded through a ring carbon), and where n is 0 or 1.

[0056] An "amide" is a chemical moiety with formula $-(R)_n-C(O)NHR'$ or $-(R)_n-NHC(O)R'$, where R and R' are independently selected from the group consisting of alkyl, cycloalkyl, aryl, heteroaryl (bonded through a ring carbon) and heterocyclic (bonded through a ring carbon), and where n is 0 or 1. An amide may be an amino acid or a peptide molecule attached to a molecule of the present invention, thereby forming a prodrug.

[0057] Any amine, hydroxy, or carboxyl side chain on the compounds of the present invention can be esterified or amidified. The procedures and specific groups to be used to achieve this end are known to those of skill in the art and can readily be found in reference sources such as Greene and Wuts, *Protective Groups in Organic Synthesis*, 3rd Ed., John Wiley & Sons, New York, N.Y., 1999, which is incorporated herein in its entirety.

[0058] The terms "purified," "substantially purified," and "isolated" as used herein refer to compounds disclosed herein being free of other, dissimilar compounds with which the compounds of the invention are normally associated in their natural state, so that the compounds of the invention comprise at least 0.5%, 1%, 5%, 10%, or 20%, and most preferably at least 50% or 75% of the mass, by weight, of a given sample.

Methods of Use

[0059] As demonstrated by the examples presented herein, the compound of formula (I) inhibits chymotrypsin-like, trypsin-like, and caspase-like proteasome activities. In contrast, Bortezomib has been shown to inhibit only chymotrypsin-like proteasome activity. See Goldberg, A. L., & Rock, K. (2002) *Nat Med* 8, 338-40 and Adams, J. (2004) *Nat Rev Cancer* 4, 349-60; both of which are incorporated herein by

reference in their entirety. It is further demonstrated that compounds of formula (I) have a different mechanism of action than bortezomib. Furthermore, the compound of formula (I) induces apoptosis in various multiple myeloma cell lines including, but not limited to, Dexamethasone-sensitive MM.1S, Dexamethasone-resistant MM.1R, RPMI-8226, OPM2, U266, and Doxorubicin-resistant Dox-40. The compound of formula I also induced apoptosis in cell lines obtained from human multiple myeloma patients that had relapsed after multiple prior therapies with Dexamethasone, Bortezomib, and thalidomide. Thus, the compound of formula (I) is effective against MM cells that are resistant to other chemotherapeutic agents, including Dexamethasone, Doxorubicin, Bortezomib/PS-341, and thalidomide.

[0060] Accordingly, in one embodiment, a method of treating a neoplastic disease that is susceptible to resistance to at least one chemotherapeutic agent is provided comprising administering to a patient, such as a human, a compound of formula (I) or a pharmaceutically acceptable salt or prodrug ester thereof. By "resistance to at least one chemotherapeutic agent," it is meant that administration of the chemotherapeutic agent to the patient does not result in significant amelioration of symptoms of the neoplastic disease. In some embodiments where the neoplastic disease is characterized by a tumor, "resistance to at least one chemotherapeutic agent" means that administration of the chemotherapeutic agent does not result in appreciable inhibition of the growth of the tumor or reduction in the size of the tumor. "Resistance to at least one chemotherapeutic agent" can also mean that when the agent is exposed to resistant tumor cells, no appreciable apoptosis is induced. By "susceptible to" resistance to at least one chemotherapeutic agent, it is meant that the neoplastic disease currently is resistant to the at least one chemotherapeutic agent or will develop resistance upon repeated administration of the chemotherapeutic agent.

[0061] The examples herein also demonstrate that compounds of formula (I) when combined with bortezomib trigger synergistic apoptosis in MM cells. Thus, a compound of formula (I) may be administered in combination with Bortezomib/PS-341 to achieve apoptosis using lower doses of each agent than if the agents were administered separately, thus reducing the toxicity of the agents. Surprisingly, these results demonstrate that a synergistic result may be obtained by administering two different proteasome inhibitors. By "synergistic," it is meant that the combination of two or more agents yield a combination index (CI) < 1.0. It has also been demonstrated that combination of the compound of formula (I) with non-proteasome inhibitor agents provide an additive effect. By "additive," it is meant that the combination of two or more agents yield a CI approximately equal to one. CI may be determined, for example, by the Chou-Talalay method according to the following equation: " $CI = (D)_1 / (Dx)_1 + (D)_2 / (Dx)_2 + (D)_1(D)_2 / (Dx)_1(Dx)_2$ ", where (D)₁ and (D)₂ are the doses of drug 1 and drug 2 that have x effect when used in combination; and (Dx)₁ and (Dx)₂ are the doses of drug 1 and drug 2 that have the same x effect when used alone.

[0062] Accordingly, in one embodiment, a method is provided for treating a neoplastic disease comprising administering two or more proteasome inhibitors in synergistic combination. Non-limiting examples of classes of proteasome inhibitors that may be combined include peptide boronate proteasome inhibitors, peptide aldehyde proteasome inhibitors, and non-peptide proteasome inhibitors. A non-limiting example of a peptide boronate proteasome inhibitor is bort-

ezomib. A non-limiting example of a peptide aldehyde proteasome inhibitor is MG-132. Non-limiting examples of non-peptide proteasome inhibitors include omuralide and the compound of formula (I). In one embodiment, at least one of the proteasome inhibitors is a compound of formula (I) or bortezomib. By administration in "combination," it is meant that the two or more agents may be found in the patient's bloodstream at the same time, regardless of when or how they are actually administered. In one embodiment, the agents are administered simultaneously. In one such embodiment, administration in combination is accomplished by combining the agents in a single dosage form. In another embodiment, the agents are administered sequentially. In one embodiment the agents are administered through the same route, such as orally. In another embodiment, the agents are administered through different routes, such as one being administered orally and another being administered i.v. In one advantageous embodiment, the pharmacokinetics of the two or more agents are substantially the same.

[0063] In one embodiment, a method is provided for treating a neoplastic disease comprising administering a compound of formula (I) in combination with another chemotherapeutic agent. In one embodiment, the other chemotherapeutic agent is dexamethasone, doxorubicin, or thalidomide. In one embodiment, the other chemotherapeutic agent is another proteasome inhibitor such as bortezomib. In one embodiment, a pharmaceutical composition is provided that combines a compound of formula (I) with the additional chemotherapeutic agent.

[0064] In some embodiments, the neoplastic disease treated by any of the methods above may be a cancer selected from breast cancer, sarcoma, leukemia, ovarian cancer, uterine cancer, bladder cancer, prostate cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, multiple myeloma, pancreatic cancer, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angiosarcoma, and brain or central nervous system (CNS) cancer. In one embodiment, the neoplastic disease is a multiple myeloma.

Pharmaceutical Compositions

[0065] In another aspect, the present disclosure relates to a pharmaceutical composition comprising physiologically acceptable surface active agents, carriers, diluents, excipients, smoothing agents, suspension agents, film forming substances, and coating assistants, or a combination thereof; and a compound or combination disclosed herein. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, Pa. (1990), which is incorporated herein by reference in its entirety. Preservatives, stabilizers, dyes, sweeteners, fragrances, flavoring agents, and the like may be provided in the pharmaceutical composition. For example, sodium benzoate, ascorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. In addition, antioxidants and suspending agents may be used. In various embodiments, alcohols, esters, sulfated aliphatic alcohols, and the like may be used as surface active agents; sucrose, glucose, lactose, starch, crystallized cellulose, mannitol, light anhydrous silicate, magnesium aluminate, magnesium methasilicate aluminate, synthetic aluminum silicate, calcium carbonate, sodium acid carbonate, calcium hydrogen phosphate, calcium carboxymethyl cellulose, and the like may be used as excipients; magnesium stearate, talc, hardened oil and the like

may be used as smoothing agents; coconut oil, olive oil, sesame oil, peanut oil, soya may be used as suspension agents or lubricants; cellulose acetate phthalate as a derivative of a carbohydrate such as cellulose or sugar, or methylacetate-methacrylate copolymer as a derivative of polyvinyl may be used as suspension agents; and plasticizers such as ester phthalates and the like may be used as suspension agents.

[0066] The term "pharmaceutical composition" refers to a mixture of a compound or combination of compounds disclosed herein with other chemical components, such as diluents or carriers. The pharmaceutical composition facilitates administration of the compound to an organism. Multiple techniques of administering a compound exist in the art including, but not limited to, oral, injection, aerosol, parenteral, and topical administration. Pharmaceutical compositions can also be obtained by reacting compounds with inorganic or organic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like.

[0067] The term "carrier" defines a chemical compound that facilitates the incorporation of a compound into cells or tissues. For example dimethyl sulfoxide (DMSO) is a commonly utilized carrier as it facilitates the uptake of many organic compounds into the cells or tissues of an organism.

[0068] The term "diluent" defines chemical compounds diluted in water that will dissolve the compound of interest as well as stabilize the biologically active form of the compound. Salts dissolved in buffered solutions are utilized as diluents in the art. One commonly used buffered solution is phosphate buffered saline because it mimics the salt conditions of human blood. Since buffer salts can control the pH of a solution at low concentrations, a buffered diluent rarely modifies the biological activity of a compound.

[0069] The term "physiologically acceptable" defines a carrier or diluent that does not abrogate the biological activity and properties of the compound.

[0070] The pharmaceutical compositions described herein can be administered to a human patient per se, or in pharmaceutical compositions where they are mixed with suitable carriers or excipient(s). Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., 18th edition, 1990.

[0071] Suitable routes of administration may, for example, include oral, rectal, transmucosal, topical, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intravenous, intramedullary injections, as well as intrathecal, direct intraventricular, intraperitoneal, intranasal, or intraocular injections. The compounds can also be administered in sustained or controlled release dosage forms, including depot injections, osmotic pumps, pills, transdermal (including electrosensory) patches, and the like, for prolonged and/or timed, pulsed administration at a predetermined rate.

[0072] The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapment or tableting processes.

[0073] Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate

processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen. Any of the well-known techniques, carriers, and excipients may be used as suitable and as understood in the art; e.g., in Remington's Pharmaceutical Sciences, above.

[0074] Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. Physiologically compatible buffers include, but are not limited to, Hanks's solution, Ringer's solution, or physiological saline buffer. If desired, absorption enhancing preparations (for example, liposomes), may be utilized.

[0075] For transcutaneous administration, penetrants appropriate to the barrier to be permeated may be used in the formulation.

[0076] Pharmaceutical formulations for parenteral administration, e.g., by bolus injection or continuous infusion, include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or other organic oils such as soybean, grapefruit or almond oils, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

[0077] For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof such as

sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[0078] Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

[0079] For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

[0080] For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0081] Further disclosed herein are various pharmaceutical compositions well known in the pharmaceutical art for uses that include intraocular, intranasal, and intraauricular delivery. Suitable penetrants for these uses are generally known in the art. Pharmaceutical compositions for intraocular delivery include aqueous ophthalmic solutions of the active compounds in water-soluble form, such as eyedrops, or in gellan gum (Shedden et al., *Clin. Ther.*, 23(3):440-50 (2001)) or hydrogels (Mayer et al., *Ophthalmologica*, 210(2):101-3 (1996)); ophthalmic ointments; ophthalmic suspensions, such as microparticulates, drug-containing small polymeric particles that are suspended in a liquid carrier medium (Joshi, A., *J. Ocul. Pharmacol.*, 10(1):29-45 (1994)); lipid-soluble formulations (Alm et al., *Prog. Clin. Biol. Res.*, 312:447-58 (1989)), and microspheres (Mordenti, *Toxicol. Sci.*, 52(1):101-6 (1999)); and ocular inserts. All of the above-mentioned references, are incorporated herein by reference in their entireties. Such suitable pharmaceutical formulations are most often and preferably formulated to be sterile, isotonic and buffered for stability and comfort. Pharmaceutical compositions for intranasal delivery may also include drops and

sprays often prepared to simulate in many respects nasal secretions to ensure maintenance of normal ciliary action. As disclosed in Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, Pa. (1990), which is incorporated herein by reference in its entirety, and well-known to those skilled in the art, suitable formulations are most often and preferably isotonic, slightly buffered to maintain a pH of 5.5 to 6.5, and most often and preferably include antimicrobial preservatives and appropriate drug stabilizers. Pharmaceutical formulations for intraauricular delivery include suspensions and ointments for topical application in the ear. Common solvents for such aural formulations include glycerin and water.

[0082] The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

[0083] In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0084] For hydrophobic compounds, a suitable pharmaceutical carrier may be a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. A common cosolvent system used is the VPD co-solvent system, which is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80TM, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied; for example, other low-toxicity nonpolar surfactants may be used instead of POLYSORBATE 80TM, the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g., polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

[0085] Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

[0086] Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes. All molecules present in an aqueous solution at the time of liposome formation are incor-

porated into the aqueous interior. The liposomal contents are both protected from the external micro-environment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. The liposome may be coated with a tissue-specific antibody. The liposomes will be targeted to and taken up selectively by the desired organ. Alternatively, small hydrophobic organic molecules may be directly administered intracellularly.

[0087] Additional therapeutic or diagnostic agents may be incorporated into the pharmaceutical compositions. Alternatively or additionally, pharmaceutical compositions may be combined with other compositions that contain other therapeutic or diagnostic agents.

Methods of Administration

[0088] The compounds or pharmaceutical compositions may be administered to the patient by any suitable means. Non-limiting examples of methods of administration include, among others, (a) administration through oral pathways, which administration includes administration in capsule, tablet, granule, spray, syrup, or other such forms; (b) administration through non-oral pathways such as rectal, vaginal, intraurethral, intraocular, intranasal, or intraauricular, which administration includes administration as an aqueous suspension, an oily preparation or the like or as a drip, spray, suppository, salve, ointment or the like; (c) administration via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, intradermally, intraorbitally, intracapsularly, intraspinally, intrasternally, or the like, including infusion pump delivery; (d) administration locally such as by injection directly in the renal or cardiac area, e.g., by depot implantation; as well as (e) administration topically; as deemed appropriate by those of skill in the art for bringing the compound of the invention into contact with living tissue.

[0089] Pharmaceutical compositions suitable for administration include compositions where the active ingredients are contained in an amount effective to achieve its intended purpose. The therapeutically effective amount of the compounds disclosed herein required as a dose will depend on the route of administration, the type of animal, including human, being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize. More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

[0090] As will be readily apparent to one skilled in the art, the useful *in vivo* dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular compounds employed, and the specific use for which these compounds are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, can be accomplished by one skilled in the art using routine pharmacological methods. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. Alternatively, acceptable *in vitro* studies can be used to establish useful doses and routes of

administration of the compositions identified by the present methods using established pharmacological methods.

[0091] In non-human animal studies, applications of potential products are commenced at higher dosage levels, with dosage being decreased until the desired effect is no longer achieved or adverse side effects disappear. The dosage may range broadly, depending upon the desired effects and the therapeutic indication. Typically, dosages may be between about 10 microgram/kg and 100 mg/kg body weight, preferably between about 100 microgram/kg and 10 mg/kg body weight. Alternatively dosages may be based and calculated upon the surface area of the patient, as understood by those of skill in the art.

[0092] The exact formulation, route of administration and dosage for the pharmaceutical compositions of the present invention can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl et al. 1975, in "The Pharmacological Basis of Therapeutics", which is hereby incorporated herein by reference in its entirety, with particular reference to Ch. 1, p. 1). Typically, the dose range of the composition administered to the patient can be from about 0.5 to 1000 mg/kg of the patient's body weight. The dosage may be a single one or a series of two or more given in the course of one or more days, as is needed by the patient. In instances where human dosages for compounds have been established for at least some condition, the present invention will use those same dosages, or dosages that are between about 0.1% and 500%, more preferably between about 25% and 250% of the established human dosage. Where no human dosage is established, as will be the case for newly-discovered pharmaceutical compounds, a suitable human dosage can be inferred from ED_{50} or LD_{50} values, or other appropriate values derived from *in vitro* or *in vivo* studies, as qualified by toxicity studies and efficacy studies in animals.

[0093] It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity or organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

[0094] Although the exact dosage will be determined on a drug-by-drug basis, in most cases, some generalizations regarding the dosage can be made. The daily dosage regimen for an adult human patient may be, for example, an oral dose of between 0.1 mg and 2000 mg of each active ingredient, preferably between 1 mg and 500 mg, e.g. 5 to 200 mg. In other embodiments, an intravenous, subcutaneous, or intramuscular dose of each active ingredient of between 0.01 mg and 100 mg, preferably between 0.1 mg and 60 mg, e.g. 1 to 40 mg is used. In cases of administration of a pharmaceutically acceptable salt, dosages may be calculated as the free base. In some embodiments, the composition is administered 1 to 4 times per day. Alternatively the compositions of the invention may be administered by continuous intravenous infusion, preferably at a dose of each active ingredient up to 1000 mg per day. As will be understood by those of skill in the

art, in certain situations it may be necessary to administer the compounds disclosed herein in amounts that exceed, or even far exceed, the above-stated, preferred dosage range in order to effectively and aggressively treat particularly aggressive diseases or infections. In some embodiments, the compounds will be administered for a period of continuous therapy, for example for a week or more, or for months or years.

[0095] Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from *in vitro* data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, *in vivo* assays or bioassays can be used to determine plasma concentrations.

[0096] Dosage intervals can also be determined using MEC value. Compositions should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

[0097] In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

[0098] The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

[0099] Compounds disclosed herein can be evaluated for efficacy and toxicity using known methods. For example, the toxicology of a particular compound, or of a subset of the compounds, sharing certain chemical moieties, may be established by determining *in vitro* toxicity towards a cell line, such as a mammalian, and preferably human, cell line. The results of such studies are often predictive of toxicity in animals, such as mammals, or more specifically, humans. Alternatively, the toxicity of particular compounds in an animal model, such as mice, rats, rabbits, or monkeys, may be determined using known methods. The efficacy of a particular compound may be established using several recognized methods, such as *in vitro* methods, animal models, or human clinical trials. Recognized *in vitro* models exist for nearly every class of condition, including but not limited to cancer, cardiovascular disease, and various immune dysfunction. Similarly, acceptable animal models may be used to establish efficacy of chemicals to treat such conditions. When selecting a model to determine efficacy, the skilled artisan can be guided by the state of the art to choose an appropriate model, dose, and route of administration, and regime. Of course, human clinical trials can also be used to determine the efficacy of a compound in humans.

[0100] The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied with a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the drug for human or veterinary administration. Such notice, for example, may be the labeling approved by the U.S. Food and Drug Administration for prescription drugs, or the approved

product insert. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

EXAMPLES

Example 1

General Procedures

[0101] Cell culture and reagents. Dex-sensitive MM.1S and Dex-resistant MM.1R human MM cell lines were obtained from Dr. Steven Rosen (Northwestern University, Chicago, Ill.). See Moalli, P.A., Pillay, S., Weiner, D., Leikin, R. & Rosen, S. T. (1992) *Blood* 79, 213-22 and Chauhan, D., Catley, L., Hideshima, T., Li, G., Leblanc, R., Gupta, D., Sattler, M., Richardson, P., Schlossman, R. L., Podar, K., Weller, E., Munshi, N. & Anderson, K. C. (2002) *Blood* 100, 2187-94; both of which are incorporated herein by reference in their entirety. RPMI-8226 and Doxorubicin (Dox)-resistant (Dox-40) cells were obtained from Dr. William Dalton (Molfit Cancer Center, Tampa, Fla.). U266 and OPM2 MM cell lines were obtained from the American Type Culture Collection (Rockville, Md.). The human tumor cell lines DU 145, HT-29, Jurkat, LoVo, MDA-MB-231, MIA PaCa-2, NCI-H292, OVCAR-3, PANC-1, and PC-3 were purchased from ATCC (Manassas, Va.). MM Cell lines were grown in RPMI-1640 media supplemented with 10% heat inactivated fetal-bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. MM cells were freshly isolated from patients relapsing after multiple prior therapies including Dexamethasone (Dex), melphalan, thalidomide or Bortezomib. MM cells were purified from patient bone marrow samples by CD138 positive selection method using CD138 (Syndecan-1) Micro Beads and the Auto MACS magnetic cell sorter (Miltenyi Biotec Inc., Auburn, Calif.). See Chauhan, D., Catley, L., Hideshima, T., Li, G., Leblanc, R., Gupta, D., Sattler, M., Richardson, P., Schlossman, R. L., Podar, K., Weller, E., Munshi, N. & Anderson, K. C. (2002) *Blood* 100, 2187-94; which is incorporated herein by reference in its entirety. Normal human skin fibroblasts CCD-27sk were obtained from ATCC and grown in DMEM supplemented with 10% heat inactivated FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine and 1 mM sodium pyruvate. Cells were treated with various concentrations of the compound of formula II (X=C1) (Nereus Pharmaceuticals, Inc, San Diego, Calif), Bortezomib or Dex (Sigma Chemical Co, St. Louis, Mo.).

[0102] Cell viability and apoptosis assays. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International Inc., Temecula, Calif.) assay, according to manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, Ind.), and as described in Chauhan, D., Catley, L., Hideshima, T., Li, G., Leblanc, R., Gupta, D., Sattler, M., Richardson, P., Schlossman, R. L., Podar, K., Weller, E., Munshi, N. & Anderson, K. C. (2002) *Blood* 100, 2187-94; which is incorporated herein by reference in its entirety. Cell Death Detection ELISAplus was utilized to quantitate cell death, as per manufacturer's instructions (Roche Applied Sciences, Indianapolis, Ind.).

Example 2

In Vitro 20S Proteasome Activity Assay

[0103] The chymotrypsin-like activity of the 20S proteasome was measured as described in Stein, R. L., Melandri, F.

& Dick, L. (1996) *Biochemistry* 35, 3899-908 and Lightcap, E. S., McCormack, T. A., Pien, C. S., Chau, V., Adams, J. & Elliott, P. J. (2000) *Clin Chem* 46, 673-83; both of which are incorporated herein by reference in their entirety. Purified human erythrocyte-derived 20S proteasome were obtained from Biomol, Plymouth Meeting, Pa. The chymotrypsin-like, caspase-like and trypsin-like activity activities of the 20S proteasome were determined using Suc-LLVY-AMC, Z-LLE-AMC (Boston Biochem, Cambridge, Mass.) and Boc-LRR-AMC (Bochem Bioscience, King of Prussia, Pa.) as peptide substrates, respectively. Fluorescence of the cleaved peptide substrate was measured using a Fluoroskan Ascent 96-well microplate reader (Thermo Electron, Waltham, Mass.). The IC_{50} values were calculated by Prism (GraphPad Software) using a sigmoidal dose-response, variable slope model. The IC_{50} values were defined as the drug concentration at which 50% of the maximal relative fluorescence is inhibited. The results, plotted in FIG. 1, indicated that the compound of formula (II) (X=Cl) inhibits all three proteasome activities, albeit at different concentrations.

Example 3

Analysis of Ex Vivo 20S Proteasome Activity in Whole Blood Cells in Mice (Single I.V. or Oral Administration)

[0104] To directly determine whether the compound of formula (II) (X=Cl) inhibits proteasome activity in vivo, the compound of formula (II) (X=Cl) was dissolved in 100% DMSO and serially diluted with 5% Solutol (Solutol® HS 15; polyethylene glycol 660 12-hydroxystearate, BASF, Shreveport, La.) yielding a final concentration of 2% DMSO. The vehicle control consisted of 2% DMSO and 98% (5% Solutol® HS15). Male Swiss-Webster mice (five per group, 20-25 grams in weight) were treated at Bolder BioPATH, Inc. (Boulder, Colo.) with various concentrations of the compound either intravenously or orally at a volume of 10 mL/kg. One group of animals was untreated to establish a baseline of proteasome activity. Ninety minutes after administration of the compound, the animals were anesthetized and blood withdrawn by cardiac puncture. Packed whole blood cells were collected by centrifugation, washed with PBS, and frozen on dry ice for determination of ex vivo proteasome activity. Chymotrypsin-like activity of the 20S proteasome in white blood cell (WBC) lysates was determined using the peptide substrate suc-LLVY-AMC. Relative Fluorescence Units (RFU) were normalized using the protein concentrations of the cell lysates. The 20S proteasome activity of the individual mice is shown in FIG. 2 with the horizontal bar representing the average activity. Baseline represents the 20S proteasome activity observed in WBC lysates prepared from untreated mice. The results, depicted in FIG. 2, indicate that the compound of formula (II) (X=Cl) inhibits chymotrypsin-like activity of 20S proteasomes in white blood cells in a dose-dependent manner. Importantly, these findings establish that the compound is orally active and inhibits proteasome activity in vivo.

Example 4

Determination of Triggered Alterations in Proteasome Activity in Mm Cells (In Vitro)

[0105] Determination of whether the compound of formula II (X=Cl) affects the proteasome activity in multiple

myeloma cells in vitro was made using a competition experiment with AdaY¹²⁵Iahx₃L₃VS. In this assay, sites that are not targeted by the compound of formula II (X=Cl) are labeled by AdaY¹²⁵Iahx₃L₃VS and visualized by autoradiography, while sites that are targeted by the compound of formula II (X=Cl) can not be seen on the autoradiogram. MM.1S cells were incubated with the compound of formula (II) (X=Cl) (7 nM) for 30 mins, 1 h, 3 h, or 6 h, and cell lysis was performed with glass beads. 60 µg of protein extracts was incubated for 2 h with the iodinated proteasome inhibitor AdaY¹²⁵Iahx₃L₃VS at 37° C. Proteins were then denatured by boiling in reducing sample buffer and separated on a 12.5% SDS-PAGE gel, followed by autoradiography. As can be seen in FIG. 3, the beta-5 (β-5) subunit of the proteasome is markedly less labeled by AdaY¹²⁵Iahx₃L₃VS in treated cells than control cells. Given that the β-5 subunit mediates the chymotrypsin-like activity, these results suggest that the compound of formula (II) (X=Cl) binds to the β-5 subunit, thereby inhibiting the chymotrypsin-like activity in MM.1S cells. Moreover, treatment of MM.1S cells with the compound (7 nM) for 6 h also decreased the labeling of the β-2 subunits (tryptic-activity) and the β-1 subunits (caspase-like activity) (data not shown).

Example 5

Determination of Triggered Alterations in Proteasome Activity in MM Cells (In Vivo)

[0106] In vivo determination of proteasome activity was conducted using a competition experiment with Dansyl-Ahx₃L₃VS, which covalently modifies all active proteasome subunits. This inhibitor contains a dansyl sulfonamide hexanoyl hapten that can be visualized by immunoblotting using antibodies against the dansyl moiety. MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 30 mins, 1 h, or 3 h, followed by 1 h incubation with 5 µM Dansyl-Ahx₃L₃VS at 37° C. Cells were lysed by incubating them for 30 mins in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40), followed by 5 min. centrifugation to remove membrane fractions, nuclei, and cell debris. 60 µg of protein extract was separated by 12.5% SDS-PAGE gel, followed by Immunoblot analysis using polyclonal anti-dansyl polyclonal Ab (1:7500, rabbit, Molecular Probes) and horseradish peroxidase coupled goat anti-rabbit secondary antibody (Southern Biotech). Blots were developed by enhanced chemiluminescence (Western Lighting, Perkin-Elmer). As can be seen in FIG. 4, treatment of MM.1S cells with the compound of formula (II) (X=Cl) decreases the dansyl-Ahx₃L₃VS-labeling of the β-5 subunits. Furthermore, the compound also decreased the dansyl-Ahx₃L₃VS-labeling of the β-1 and β-2 subunits, albeit at higher concentrations: 1 nM and 20 nM, respectively. In contrast, treatment of MM.1S cells with even higher doses of Bortezomib does not inhibit the β-2 subunits (data not shown). Taken together, these findings demonstrate the ability of the compound of formula (II) (X=Cl) to inhibit all three proteasome activities in MM cells.

Example 6

Effect on MM Cell Viability

[0107] Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International Inc., Temecula, Calif.) assay, according to manufacturer's instructions (Roche Molecular Biochemicals,

Indianapolis, Ind.), and as described in Chauhan, D., Catley, L., Hideshima, T., Li, G., Leblanc, R., Gupta, D., Sattler, M., Richardson, P., Schlossman, R. L., Podar, K., Weller, E., Munshi, N. & Anderson, K. C. (2002) *Blood* 100, 2187-94; which is incorporated herein by reference in its entirety. Cell viability after treatment of MM.1S (■), Dex-resistant MM.1R (□), RPMI-8226 (●), Doxorubicin-resistant Dox-40 (◆), OPM2 (○), and U266 (◇) cells with the compound of formula (II) (X=Cl) for 24 h is illustrated in FIG. 5A. Results are mean±SD from three independent experiments (P<0.005; n=3 for all cell lines). A dose-dependent significant decrease in cell viability in all cell lines was observed (IC₅₀ range 7-24 nM).

[0108] Cell viability was also assessed on purified patient MM cells. Freshly isolated tumor cells from nine MM patients relapsing after multiple prior therapies including Dex, Bortezomib, and thalidomide were treated with the compound of formula (II) (X=Cl) (10 nM) for 24 h and analyzed for apoptosis. As seen in FIG. 5B, significant apoptosis was observed in these cells as measured by DNA fragmentation assays (P<0.005; n=2). Plotted values are the mean±SD of triplicate samples. Importantly, 4 of 9 patients examined were refractory to Bortezomib therapy, and 5 patients were resistant to Thalidomide and Dex therapies. These data suggest that 1) the compound of formula (II) (X=Cl) induces apoptosis in MM cells sensitive and resistant to conventional and Bortezomib therapies; and 2) IC₅₀ of the compound for MM cells is within the nanomolar concentration.

Example 7

Effect on Bone Marrow Stromal Cell (BMSCs) Viability

[0109] MM cells predominantly localize in the bone marrow microenvironment and their interaction with BMSCs induces production of cytokines which mediate growth of MM cells, as well as protect against drug-induced apoptosis. See Anderson, K. C. (2003) *Cancer* 97, 796-801; which is incorporated herein by reference in its entirety. Therefore, the effect of the compound of formula (II) (X=Cl) on five patient MM-derived BMSCs was determined. As seen in FIG. 6, treatment of BMSCs (Patient#1-5) with the compound of formula (II) (X=Cl) (20 nM) for 24 h does not induce apoptosis in these cells, as evidenced by DNA fragmentation assay. Positive control shown is an internal control for the assay. Purified MM cells (CD138+) from two of the five MM patient were also examined within the same experiments. Results are mean±SD from triplicate samples. The compound triggered a significant (10-12 fold) increase in apoptosis of purified (CD138-positive) patient MM cells. These results suggest that the compound of formula (II) (X=Cl) acts directly on MM cells, but not BMSCs.

Example 8

Effect of Recombinant Human Interleukin-6 (rhIL-6) and Recombinant Human Insulin-Like Growth Factor-1 (rhIGF-1) Anti-Apoptosis

[0110] Adhesion of MM cells to BMSCs induces IL-6 and IGF-1 secretion from BMSCs, which not only regulates the growth of MM cells, but also protects against chemotherapy. See Hardin, J., MacLeod, S., Grigorieva, I., Chang, R., Barlogie, B., Xiao, H. & Epstein, J. (1994) *Blood* 84, 3063-70 and

Chauhan, D., Kharbada, S., Ogata, A., Urashima, M., Teoh, G., Robertson, M., Kufe, D. W. & Anderson, K. C. (1997) *Blood* 89, 227-234; both of which are incorporated herein by reference in their entirety. Thus, whether rhIL-6 or rhIGF-1 inhibits apoptosis in MM cells induced by the compound of formula (II) (X=Cl) was evaluated. MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) or Dex (0.5 μM) for 24 h, in the presence and absence of rhIL-6 (10 ng/ml) or rhIGF-1 (50 ng/ml). At 24 h cells were harvested and viability analyzed by MTI assays. As seen in FIG. 7, the median cell viability was 47±2.3% after treatment with the compound alone; 51.2±3.2% with the compound+rhIL-6 (P=0.26, Wilcoxon test), and 50.3%±2.0% with the compound+rhIGF-1 (P=0.28). Median viability was 51±2.1% after treatment with Dex and 92±5.5% for Dex+rhIL-6 (P=0.05, as determined by one-sided Wilcoxon rank-sum test). Results are mean±SD of three independent experiments. These findings suggest that neither IL-6 nor IGF-1 block the anti-MM activity of the compound of formula (II) (X=Cl). In contrast and as in other studies, both IL-6 and IGF-1 block Dex-induced decreased MM.1S cell viability. See Chauhan, D., Hideshima, T. & Anderson, K. C. (2003) *Int J Hematol* 78, 114-20 and Mitsiades, C. S., Mitsiades, N., Poulaki, V., Schlossman, R., Akiyama, M., Chauhan, D., Hideshima, T., Treon, S. P., Munshi, N. C., Richardson, P. G. & Anderson, K. C. (2002) *Oncogene* 21, 5673-83; both of which are incorporated herein by reference in their entirety. Thus, the data suggests that the compound of formula (II) (X=Cl) overcomes the growth and protective effects of IL-6 and IGF-1 on MM cells, and indicate distinct mechanisms of action for the compound and Dex against MM cells. Reports that high serum levels of IL-6 contribute to clinical chemoresistance and treatment failure, coupled with the ability of the compound of formula (II) (X=Cl) to induce MM cell apoptosis even in the presence of IL-6 or IGF-1, suggest that the compound may overcome drug resistance in patients with advanced MM. See Kyrstons, M. C., Dedoussis, G., Baxevanis, C., Stamatiou, M. & Maniatis, A. (1996) *Br J Haematol* 92, 420-422; which is incorporated herein by reference in its entirety.

Example 9

Effect on Vascular Endothelial Growth Factor (VEGF) Induced Migration of MM Cells

[0111] VEGF is elevated in the bone marrow microenvironment and triggers migration, growth, and angiogenesis in MM cells. See Podar, K., Tai, Y. T., Lin, B. K., Narsinhani, R. P., Sattler, M., Kijima, T., Salgia, R., Gupta, D., Chauhan, D. & Anderson, K. C. (2002) *J Biol Chem* 277, 7875-81; which is incorporated herein by reference in its entirety. Thus, whether the compound of formula (II) (X=Cl) alters VEGF-induced migration of MM cells was evaluated. VEGF induced migration was examined in the presence or absence of the compound (7 or 10 nM). Cell migration was assayed as described previously in Podar, K., Tai, Y. T., Davies, F. E., Lentzsch, S., Sattler, M., Hideshima, T., Lin, B. K., Gupta, D., Shima, Y., Chauhan, D., Mitsiades, C., Raju, N., Richardson, P. & Anderson, K. C. (2001) *Blood* 98, 428-35; which is incorporated herein by reference in its entirety. As shown in FIG. 8, the compound of formula (II) (X=Cl) significantly (P<0.05) decreases VEGF-induced migration of MM.1S MM cells. These findings indicate that the compound may nega-

tively regulate both homing of MM cells to the bone marrow and their egress into the peripheral blood.

Example 10

Effect on Bcl2-Mediated Protective Effects

[0112] Bcl2 confers resistance to conventional therapies in cancer cells, including MM. See Cory, S. & Adams, J. M. (2002) *Nat Rev Cancer* 2, 647-56 and Gazitt, Y., Fey, V., Thomas, C. & Alvarez, R. (1998) *Int J Oncol* 13, 397-405; both of which are incorporated herein by reference in their entirety. Bcl2 can modestly attenuate Bortezomib-induced apoptosis. Thus, whether ectopic expression of Bcl2 in MM.1S cells affects responsiveness to the compound of formula (II) (X=Cl) was evaluated. MM.1S cells were stably transfected with Bcl2 construct and analyzed for alterations in cell viability using an MTT assay. As seen in FIG. 9, the compound of formula (II) (X=Cl) significantly decreases cell viability of Bcl2-transfected MM.1S cells ($P < 0.005$) in a dose-dependent manner. Nonetheless, the compound induced $15 \pm 1.1\%$ less cell death in Bcl2-transfected cells compared to empty vector-transfected MM.1S cells. Results are mean \pm SD of three independent experiments. These findings suggest that the compound can overcome Bcl2-mediated protection.

Example 11

In Vivo Evaluation in Murine Tumor Model

[0113] Six-week-old triple immune deficient beige-nude-kid (BNX) mice were obtained from Frederick Cancer Research and Development Center (Frederick, Md.). All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Dana-Farber Cancer Institute. Mice were observed daily for signs of toxicity. Terminal bleeding was done under anesthesia using isoflurane inhalation, and animals were sacrificed by CO_2 asphyxiation. To determine the in vivo anti-MM activity of the compound of formula (II) (X=Cl), 21 BNX mice were inoculated subcutaneously in the flank with 3×10^5 RPMI 8226 MM cells in 100 μl of RPMI-1640 media. When tumors became measurable, mice were assigned to treatment groups receiving the compound of formula (II) (X=Cl) 0.25 mg/kg ($n=7$), 0.5 mg/kg ($n=7$), or to control groups ($n=7$) receiving the vehicle only. Drug treatment was started after the development of measurable tumor. The drug (0.25 mg/kg or 0.5 mg/kg) was given orally twice a week. Serial caliper measurements of perpendicular diameters were done every other day to calculate tumor volume, using the following formula: $4/24 \times (\text{shortest diameter})^2 \times (\text{longest diameter})$. Animals were sacrificed if the tumor was ≥ 2 cm or necrotic. For tumor growth studies, 7 mice were used in each group.

[0114] As seen in FIGS. 10A-C, treatment of tumor bearing mice with the compound of formula (II) (X=Cl), but not with vehicle alone, significantly inhibits MM tumor growth and prolongs survival of these mice. All mice in the control group developed progressive tumors, whereas complete regression of tumors were observed in 70% of treated mice. The mouse on the upper panel of FIG. 10B received oral doses of vehicle alone, whereas the mouse on the lower panel received the compound of formula (II) (X=Cl) (0.25 mg/kg). The left panels in FIG. 10B are enlargements of subcutaneous plasmacytomas growing on the right flanks of the mice. Survival was evaluated from the first day of treatment until death; mice were sacrificed when their tumor diameters reached 2 cm or

they became moribund (FIG. 10C). Moreover, no neurological behavioral changes were observed even after 12 weeks of treatment. The concentrations of the compound administered were well tolerated by mice, without evidence of weight loss. Mice in both untreated and treated group were weighed every week. The average changes in the mice body weight are shown in FIG. 10D.

[0115] Analysis at day 300 showed no recurrence of tumor in 57% of the compound of formula (II) (X=Cl)-treated mice (FIG. 10C). In addition, histologic analysis performed on the inoculation sites confirmed the disappearance of plasma cells in the compound of formula (II) (X=Cl)—versus vehicle-treated mice (FIG. 10E, left and right panels, respectively). These data show that the compound is orally active; inhibits MM tumor growth in vivo; and prolongs survival.

Example 12

Comparative Analysis of In Vivo Antitumor Activity

[0116] To compare the in vivo activity of the compound of formula (II) and Bortezomib, the mice models as described above were treated with the compound of formula (II) (X=Cl) (0.15 mg/kg i.v.) or Bortezomib (1.0 mg/kg i.v.) twice weekly. Both agents significantly reduced the tumor progression ($p < 0.01$) and prolonged survival ($p = 0.0137$) (FIGS. 10F and 10G).

Example 13

Mechanisms Mediating Anti-MM Activity

[0117] Mitochondria play a critical role in apoptosis induction during stress. See Bossy-Wetzel, E. & Green, D. R. (1999) *Mutat Res* 434, 243-51 and Chaudhan, D. & Anderson, K. C. (2003) *Apoptosis* 8, 337-43; both of which are incorporated herein by reference in their entirety. Serum starved MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 12 h and incubated with CMXRos for the last 20 min; stained with lipophilic cationic dye CMXRos (Mitotracker Red) (Molecular Probes, Eugene, Oreg.) in phosphate-buffered saline (PBS) for 20 mins at 37°C ; and analyzed by flow cytometry to assay for alterations in Ψm (mitochondrial membrane potential). Superoxide (O_2^-) production was measured by staining cells with membrane permeable dye dihydroethidium (HE) for the last 15 min. Superoxide anions oxidize HE to fluorescent ethidium, permitting analysis by flow cytometry.

[0118] As seen in FIGS. 11A and 11B, the compound of formula (II) (X=Cl) decreases $\Delta\Psi\text{m}$, evidenced by an increased number of CMXRos negative cells ($P < 0.005$, $n=2$), and triggers O_2^- production in MM.1S cells. Results are mean \pm SD of two independent experiments. Alterations in $\Delta\Psi\text{m}$ are associated with release of mitochondrial proteins cyto-c and Smac to the cytosol, thereby triggering caspase 9 and caspase-3. See Du, C., Fang, M., Li, Y., Li, L. & Wang, X. (2000) *Cell* 102, 33-42 and Liu, X., Naekyung Kim, C., Yang, J., Jemerson, R. & Wang, X. (1996) *Cell* 86, 147-157; both of which are incorporated herein by reference in their entirety.

[0119] As seen in FIG. 11C, treatment of MM.1S cells with compound of formula (II) (X=Cl) triggers a decrease in mitochondrial cyto-c (upper, left panel) and smac (upper, right panel), and a concurrent increase of these proteins in the cytosolic fractions (middle, left and right panels, respectively). Reprobing the immunoblots with anti-Hsp60 (lower, left panel) and anti-tubulin (lower, right panel) Abs confirms

purity of mitochondrial extracts and equal protein loading. Release of mitochondrial apoptogenic proteins cyto-c and Smac/DIABLO induce activation of caspases-9 and -3. MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 24 h and harvested; mitochondrial and cytosolic protein fractions were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with anti-cyto-c (upper panel) or anti-Smac (middle panel) Abs. As a control for equal loading of proteins and purity of mitochondrial fractions, filters were also reprobed with anti-tubulin (lower right panel) and anti-Hsp60 Abs (lower left panel), respectively. Blots are representative of three independent experiments.

[0120] MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 24 h and harvested; cytosolic proteins were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with anti-caspase-8 Abs and anti-caspase-9 Abs. As seen in FIG. 11D, treatment of MM.1 S cells with the compound of formula (II) (X=Cl) induces proteolytic cleavage of caspase-9. Moreover, the compound also activates caspase-8 (FIG. 11E). Both caspase-9 (mitochondria-dependent) and caspase-8 (mitochondria-independent) are known to proteolytically cleave and activate a common downstream effector caspase-3, resulting in PARP cleavage. See Miller, L. K. (1999) *Trends Cell Biol* 9, 323-8; which is incorporated herein by reference in its entirety. Thus, MM.1S or MM.1R MM cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 24 h and assessed for apoptosis by both PARP and caspase-3 cleavage assays. Total protein lysates were subjected to SDS-PAGE analysis. Immunoblot analysis of the lysates was performed with anti-PARP (upper panel) or anti-caspase-3 (lower panel) Abs. 'FL' indicates 'full length' and 'CF' denotes cleaved fragment. This data further shows that the compound of formula (II) (X=Cl) triggers caspase-3 and PARP cleavage (FIG. 11F).

[0121] Immunoblot analysis was performed using antibodies to cytochrome-c, Smac, Caspase-8, -9, or -3 (Cell Signaling, Beverly, Mass.), tubulin (Sigma, St. Louis, Mo.), PARP, Hsp60, or Bax (BD Bioscience Pharmingen, San Diego, Calif.). Blots were developed by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, Ill.).

Example 14

Mechanistic Differences of MM Cell Apoptosis Compared to Bortezomib

[0122] MM.1S cells were treated with the compound of formula (II) (X=Cl) or Bortezomib in the presence or absence of caspase-9 inhibitor (LEHD-FMK), caspase-8 inhibitor (IETD-fmk) or caspase-3 inhibitor (Z-Val-Ala-Asp-fluoromethylketone, z-VAD-fmk). As seen in FIG. 12A, caspase-3 inhibition markedly abrogates both the compound of formula (II) (X=Cl) and Bortezomib-induced apoptosis. Results are mean±SD of four independent experiments (P<0.004). Blockade of caspase-8 led to a significant decrease in cell death triggered by the compound of formula (II) (X=Cl) (P<0.005, n=4), whereas inhibition of caspase-9 only moderately blocked decreased viability in MM.1S cells triggered by the compound. In contrast, Bortezomib-induced decrease in viability of MM.1S cells is equally blocked in the presence of either caspase-8 or caspase-9 inhibitor (P<0.005). Together, these data suggest that caspase-8 and caspase-9 activation equally contribute during Bortezomib-triggered cell death, whereas apoptosis triggered by the compound of formula (II) (X=Cl) proceeds primarily via caspase-8 signaling pathway.

[0123] These biochemical data were confirmed by genetic studies using dominant-negative (DN) strategies. MM.1S cells were also transiently transfected using Cell line Nucleofecto kit V, according to the manufacturer's instructions (Amata Biosystems, Germany), with vector alone, DN-caspase-8, DN-caspase-9, or DN-FADD and cotransfected with vector containing green fluorescence protein (GFP) alone. Following transfections, GFP-positive cells were selected by flow cytometry, treated with the compound of formula (II) (X=Cl) or Bortezomib, and analyzed for viability. Treatment of DN-caspase-8-transfected MM cells with the compound of formula (II) (X=Cl) (IC50, 7 nM) markedly increased survival of these cells, compared to the cells transfected with DN-caspase-9 (FIG. 12B). In contrast, treatment of either DN-caspase-8 or DN-caspase-9-transfected MM.1S cells with Bortezomib (IC50, 5 nM) increased the survival to a similar extent. The functional specificity of DN-caspase-8 and DN-caspase-9 was confirmed by treatment of MM.1S cells with known inducers of caspase-9 (Dex) and caspase-8 in these cells (anti-Fas MoAb) (Chauhan et al., 1997) (FIG. 12C). These data suggest that (1) compound of formula (II) (X=Cl)-induced MM cell apoptosis is predominantly mediated by caspase-8; and (2) Bortezomib-induced apoptosis requires both caspase-8 and caspase-9 activation.

[0124] It was next determined whether inhibition of an upstream signaling pathway that leads to caspase-8 activation affects the response to the compound of formula (II) (X=Cl) or Bortezomib. The Fas-associated death domain (FADD) protein is an important part of the deactivating signaling complexes (DISCs) that assemble upon engagement of TNF receptor family members, such as Fas, resulting in proteolytic processing and autoactivation of pro-caspase-8. Since both the compound of formula (II) (X=Cl) and Bortezomib trigger caspase-8 activation, the role of FADD during this event in MM cells was evaluated using DN-FADD. Blockade of FADD with DN-FADD significantly attenuated compound of formula (II) (X=Cl)-induced cytotoxicity compared to the empty vector-transfected MM.1S cells (42%±2.0% viable cells in vector-transfected cells versus 76%±5.1% viable cells in DN-FADD-transfected cells; p<0.05) (FIG. 12D). DN-FADD decreased compound of formula (II) (X=Cl)-induced caspase-8 activation; however, minimal caspase-8 activation was still noted (data not shown), which may be due to upstream activators of caspase-8 other than FADD. Importantly, treatment of DN-FADD-transfected MM.1S cells with Bortezomib resulted in only a 16% increase in survival compared to vector-transfected cells (39%±2.4% viable cells in vector-transfected cells versus 55%±4.1% viable cells in DN-FADD-transfected cells; p<0.05) (FIG. 12D). These data, coupled with caspase-8 or caspase-9 inhibition studies, suggest that the compound of formula (II) relies more on FADD-caspase-8 signaling axis than does Bortezomib, further confirming differential mechanism of action of the compound of formula (II) versus Bortezomib in MM cells.

[0125] Previous studies have established that Bax induces mitochondrial apoptotic pathway. See Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B. & Korsmeyer, S. J. (2001) *Science* 292, 727-30 and Lei, K., Nimmual, A., Zong, W. X., Kennedy, N. J., Flavell, R. A., Thompson, C. B., Bar-Sagi, D. & Davis, R. J. (2002) *Mol Cell Biol* 22, 4929-42; both of which are incorporated herein by reference in their entirety. Thus, whether MM cell apoptosis induced by the compound of formula (II) (X=Cl) correlates

with alterations in Bax was evaluated. MM.1S MM cells were treated with either the compound of formula (II) (X=Cl) or Bortezomib and mitochondrial protein extracts were subjected to immunoblot analysis with anti-Bax or anti-Hsp60 Abs. As seen in FIG. 12E, the compound of formula (II) (X=Cl) induces little, if any increase in Bax levels in mitochondria. Blots are representatives of three independent experiments. Importantly, Bortezomib triggers a significant accumulation of Bax in mitochondria.

[0126] Mouse embryonic fibroblast (MEFs) carrying wild-type Bax or knock-outs were treated with the compound of formula (II) (X=Cl) or Bortezomib for 48 h and analyzed for cell viability by MTT assays. As seen in FIG. 12F, the compound of formula (II) (X=Cl) decreases viability in both Bax (WT) and Bax (knock-out), whereas deletion of Bax confers significant resistance to Bortezomib. Results are mean±SD of three independent experiments (P<0.05). These data show the differential requirement of Bax during apoptosis induced by the compound of formula (II) (X=Cl) and Bortezomib and suggest distinct mechanism of action of these agents.

Example 15

Differential Effects on Normal Lymphocytes as Compared to Bortezomib

[0127] Bortezomib therapy is associated with toxicity in patients. Thus, the effects of the compound of formula (II) (X=Cl) and Bortezomib on normal cells was compared. Lymphocytes from five healthy donors were treated with various concentrations (0-20 nM) of the compound of formula (II) (X=Cl) or Bortezomib (0-20 nM) for 72 h and analyzed for cytotoxicity by an MTT assay. As seen in FIG. 13, the compound of formula (II) (X=Cl) does not significantly decrease the survival of normal lymphocytes (P=0.27 from J-T trend test), even at higher doses (20 nM). Results are the mean±SD of three independent experiments. In contrast, Bortezomib significantly decreases the viability of lymphocytes even at lower concentrations of 6-10 nM. Of note, IC₅₀ of patient MM cells is reached at concentrations of the compound of formula (II) (X=Cl) that have no effect on normal lymphocytes, whereas IC₅₀ of Bortezomib for MM cells triggers 50% decrease in viability of normal lymphocytes. Together, these data suggest that the compound of formula (II) (X=Cl) has selective anti-MM activity; and particularly, it is less toxic to normal cells than Bortezomib.

[0128] Whether the compound of formula (II) or Bortezomib alters proteasome activity in normal lymphocytes and skin fibroblasts was also examined. Both the compound of formula (II) (X=Cl) and Bortezomib significantly inhibited proteasome activity in these cells: 20 nM of the compound of formula (II) (X=Cl) or Bortezomib triggered 99% or 59±1% inhibition of Chymotrypsin-like proteasome activity, respectively (data not shown). Thus, although 20 nM of the compound of formula (II) (X=Cl) did not trigger significant cytotoxicity in normal lymphocytes, it reduced Chymotrypsin-like proteasome activity in these cells. Similarly, treatment of normal CCD-27sk fibroblasts at the IC₅₀ for the compound of formula (II) (X=Cl) (317 nM) or Bortezomib (15 nM) also inhibited proteasome activity (data not shown).

Example 16

Differential Effects on Bcl-2-Overexpressing MM Cells as Compared to Bortezomib

[0129] During apoptosis Bax neutralizes the antiapoptotic function of Bcl-2, thereby facilitating the cyto-c release and

caspase-9 activation. Bcl-2 also confers drug resistance in cancer cells, including MM, and provides partial protection against Bortezomib-induced killing. Therefore, whether ectopic expression of Bcl-2 in MM.1S cells affects the ability of the compound of formula (II) or Bortezomib to trigger cytotoxicity and postmitochondrial apoptotic signaling in MM cells was evaluated. Overexpression of Bcl-2 promoted a modest increase in viability of cells treated with both agents: for the compound of formula (II) (X=Cl), 50%±2.6% viability in Bcl-2-transfected cells versus 39%±1.5% viability in vector-transfected cells (p<0.05); and for Bortezomib, 61%±2.9% viability in Bcl-2-transfected cells versus 40%±2.1% viability in vector-transfected cells (p<0.05) (FIG. 14A). The increased survival of Bcl-2 transfectants in response to Bortezomib was greater (21%) than that in response to the compound of formula (II) (X=Cl) (11%) (p<0.04; n=3) (FIG. 14A). Moreover, Bortezomib triggered significant caspase-9 cleavage in control vector-transfected cells, which is markedly attenuated (3-fold decrease by densitometry) in Bcl-2-transfected cells; in contrast, compound of formula (II) (X=Cl)-induced caspase-9 cleavage is minimally affected by Bcl-2 overexpression (FIG. 14B). These findings, together with the viability results, suggest that Bcl-2 provides more protection against Bortezomib than the compound of formula (II).

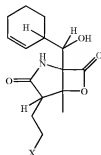
Example 17

Combination Treatment

[0130] As seen in FIG. 15, treatment of MM.1S or MM.1R MM cells with the compound of formula (II) (X=Cl) in combination with Bortezomib for 24 h induces synergistic growth inhibition. Results are mean±SD of three independent experiments (P<0.005). The interaction between anti-MM agents formula (II) (X=Cl) and Bortezomib was analyzed using isobologram analysis with "CalcuSyn" software program (Biosoft, Ferguson, Mo. and Cambridge, UK). Data from cell viability assay (MTT) were expressed as fraction of cells with growth affected (FA) in drug-treated versus untreated cells. The CalcuSyn program is based upon the Chou-Talalay method according to the following equation: $CI = (D1)/(Dx)1 + (D2)/(Dx)2 + (D1)(D2)/(Dx)1(Dx)2$, where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have x effect when used in combination; and (Dx)1 and (Dx)2 are the doses of drug 1 and drug 2 that have the same x effect when used alone. When CI=1, this equation represents the conservation isobologram and indicates additive effects. CI values of <1.0 indicate synergism. A combination index (CI) of <1.0 was obtained for Bortezomib+NPI-0052, indicating synergism. Moreover, maximal anti-MM activity was observed when given concomitantly, rather than other treatment schedules. Low doses of combined compound of formula (II) (X=Cl) and Bortezomib does not significantly affect viability of normal PBMCs (data not shown). Combination therapy with Bortezomib and the compound of formula (II) (X=Cl) therefore may: 1) allow use of sub-toxic concentrations of each agent; 2) delay or prevent development of drug-resistance; and 3) permit escalating synergistic doses of these agents to increase the apoptotic threshold.

What is claimed is:

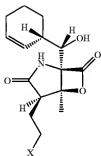
1. A method of treating a neoplastic disease, comprising: administering to a patient afflicted with the neoplastic disease a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:



wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine, and wherein the neoplastic disease is susceptible to resistance to at least one other chemotherapeutic agent.

2. The method of claim 1, wherein X is chlorine.

3. The method of claim 1, wherein the compound of formula (I) has the structure of formula (II):



4. The method of claim 1, wherein the neoplastic disease is cancer.

5. The method of claim 4, wherein the cancer is selected from the group consisting of breast cancer, sarcoma, leukemia, ovarian cancer, ureteral cancer, bladder cancer, prostate cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, multiple myeloma, pancreatic cancer, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, and brain or central nervous system (CNS) cancer.

6. The method of claim 5, wherein the cancer is selected from the group consisting of multiple myeloma, colorectal carcinoma, prostate carcinoma, breast adenocarcinoma, non-small cell lung carcinoma, and an ovarian carcinoma or melanoma.

7. The method of claim 6, wherein the cancer is a multiple myeloma.

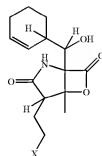
8. The method of claim 1, wherein the patient is a human.

9. The method of claim 1, wherein the other chemotherapeutic agent is selected from the group consisting of dexamethasone, doxorubicin, and thalidomide.

10. The method of claim 1, wherein the other chemotherapeutic agent is a proteasome inhibitor.

11. The method of claim 10, wherein the other chemotherapeutic agent is bortezomib.

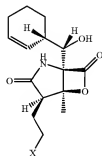
12. A method of treating a neoplastic disease, comprising administering to a patient inflicted with the neoplastic disease a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:



wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine, in combination with at least one additional chemotherapeutic agent.

13. The method of claim 12, wherein X is chlorine.

14. The method of claim 12, wherein the compound of formula (I) has the structure of formula (II):



15. The method of claim 12, wherein the neoplastic disease is cancer.

16. The method of claim 15, wherein the cancer is selected from the group consisting of breast cancer, sarcoma, leukemia, ovarian cancer, ureteral cancer, bladder cancer, prostate cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, multiple myeloma, pancreatic cancer, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, and brain or central nervous system (CNS) cancer.

17. The method of claim 16, wherein the cancer is selected from the group consisting of multiple myeloma, colorectal carcinoma, prostate carcinoma, breast adenocarcinoma, non-small cell lung carcinoma, and an ovarian carcinoma or melanoma.

18. The method of claim 17, wherein the cancer is a multiple myeloma.

19. The method of claim 12, wherein the patient is a human.

20. The method of claim 12, wherein the other chemotherapeutic agent is selected from the group consisting of dexamethasone, doxorubicin, and thalidomide.

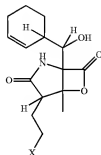
21. The method of claim 12, wherein the other chemotherapeutic agent is a proteasome inhibitor.

22. The method of claim 21, wherein the other chemotherapeutic agent is bortezomib.

23. The method of claim 12, wherein the combination is synergistic.

24. The method of claim 12, wherein the combination is additive.

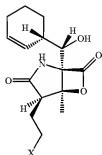
25. A pharmaceutical composition, comprising:
a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:



wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine; and
at least one additional chemotherapeutic agent.

26. The composition of claim 25, wherein X is chlorine.

27. The composition of claim 25, wherein the compound of formula (I) has the structure of formula (II):



28. The composition of claim 25, wherein the other chemotherapeutic agent is selected from the group consisting of dexamethasone, doxorubicin, and thalidomide.

29. The composition of claim 25, wherein the other chemotherapeutic agent is a proteasome inhibitor.

30. The composition of claim 29, wherein the other chemotherapeutic agent is bortezomib.

31. A method of treating a neoplastic disease, comprising administering to a patient afflicted with the neoplastic disease a synergistic combination of at least two proteasome inhibitors.

32. The method of claim 31, wherein the neoplastic disease is cancer.

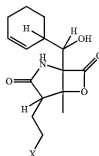
(I) 33. The method of claim 32, wherein the cancer is selected from the group consisting of breast cancer, sarcoma, leukemia, ovarian cancer, uretal cancer, bladder cancer, prostate cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, multiple myeloma, pancreatic cancer, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, and brain or central nervous system (CNS) cancer.

34. The method of claim 33, wherein the cancer is selected from the group consisting of multiple myeloma, colorectal carcinoma, prostate carcinoma, breast adenocarcinoma, non-small cell lung carcinoma, and an ovarian carcinoma or melanoma.

35. The method of claim 34, wherein the cancer is a multiple myeloma.

36. The method of claim 31, wherein the patient is a human.

37. The method of claim 31, wherein at least one of the proteasome inhibitors is selected from the group consisting of bortezomib and the compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:



wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine.

* * * * *



US 20090148445A1

(19) **United States**(12) **Patent Application Publication**
Bonavida et al.(10) **Pub. No.: US 2009/0148445 A1**(43) **Pub. Date: Jun. 11, 2009**(54) **METHODS OF SENSITIZING CANCER TO
THERAPY-INDUCED CYTOTOXICITY**(75) **Inventors:** **Benjamin Bonavida**, Los Angeles,
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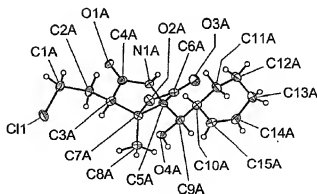
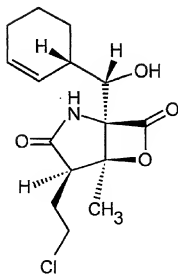
§ 371 (c)(1),
(2), (4) Date: **Feb. 19, 2009**

Related U.S. Application Data(60) Provisional application No. 60/733,965, filed on Nov.
4, 2005, provisional application No. 60/840,811, filed
on Aug. 28, 2006.**Publication Classification**(51) **Int. Cl.****A61K 39/395** (2006.01)**A61K 31/407** (2006.01)**A61K 31/69** (2006.01)**A61P 35/00** (2006.01)(52) **U.S. Cl. 424/133.1; 514/421; 514/64**

(57)

ABSTRACT

The present application demonstrates that Salinosporamide A can be used to sensitize cancer cells to cancer therapy. Furthermore, the present application demonstrates that Salinosporamide A acts as a therapeutic agent to kill or inhibit cancer cells after sensitization of the cells by an antibody or other chemosensitizing reagents. The cancer cells can be either therapy-sensitive or therapy resistant. The present application further demonstrates that Salinosporamide A induces the expression of Raf kinase inhibitor protein (RKIP) and PTEN, tumor suppressor proteins, and inhibits the expression of YY1, a transcriptional regulator protein over-expressed in cancer cells and also inhibits the growth factor pleiotrophin (PTN).



**ORTEP plot of the final X-ray
structure of NPI-0052 depicting
the absolute stereochemistry**

Fig. 1

NPI-0052 sensitizes B-NHL Ramos cell line
to CDDP-induced apoptosis

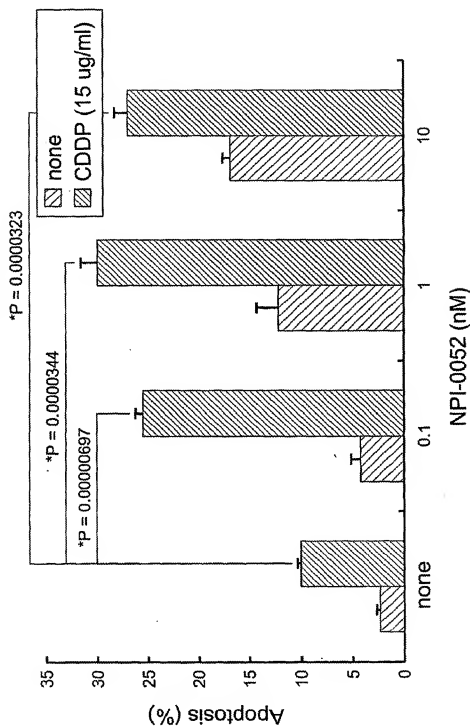


Fig. 2a

NPI-0052 sensitizes B-NHL Daudi cell line
to CDDP-induced apoptosis

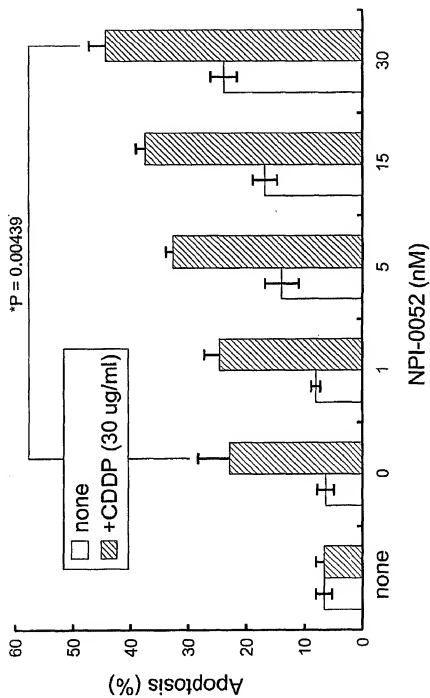


Fig. 2b

NPI-0052 sensitizes B-NHL Rituximab resistant
Daudi RR1 cell line to CDDP-induced apoptosis

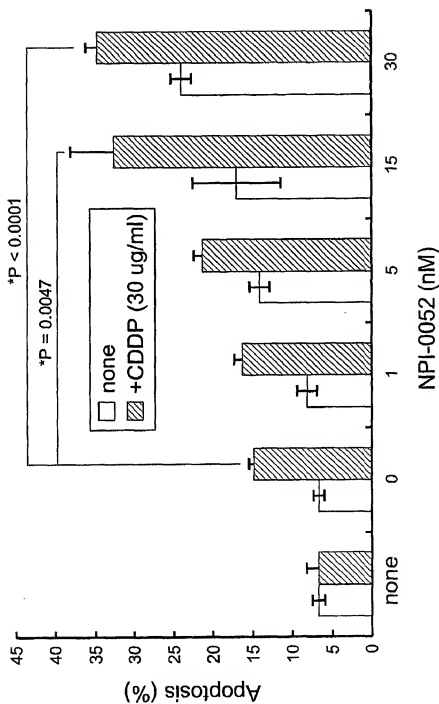


Fig. 3a

Combination between NPI-0052 and Velcade-induced sensitization to B-NHL Daudi WT cells to CDDP-induced apoptosis

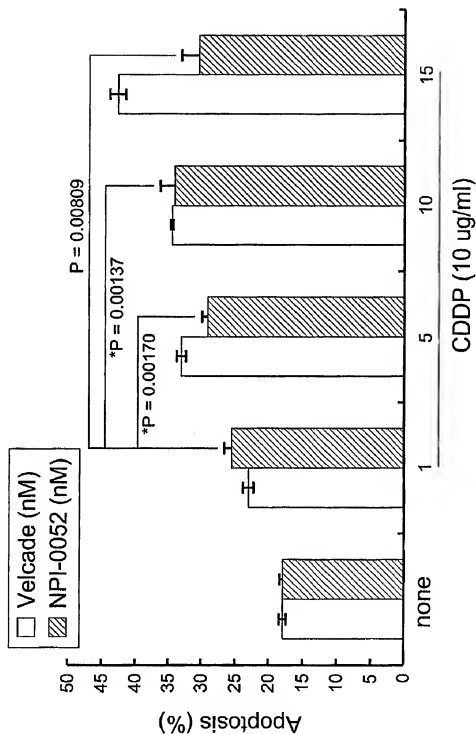


Fig. 3b

Combination between NPI-0052 and DHMEQ induced sensitization to B-NHL Daudi WT cells to CDDP-induced apoptosis

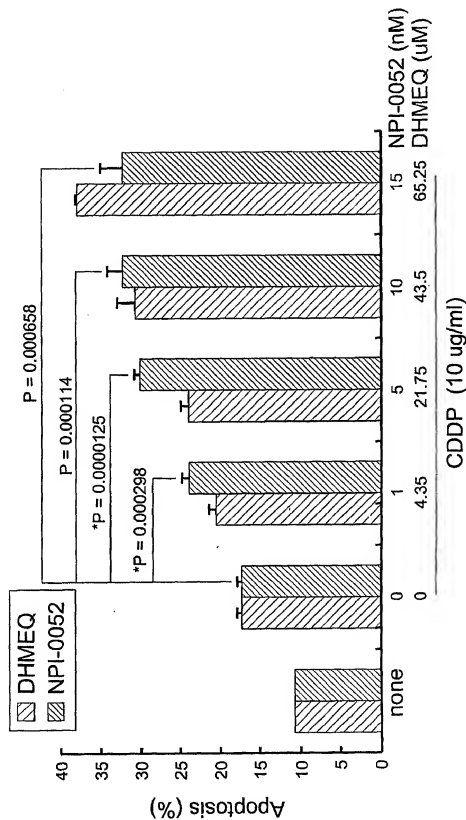


Fig. 4a

Combination between NPI-0052 and Velcade on
CDDP-induced apoptosis in Daudi RR1 cells

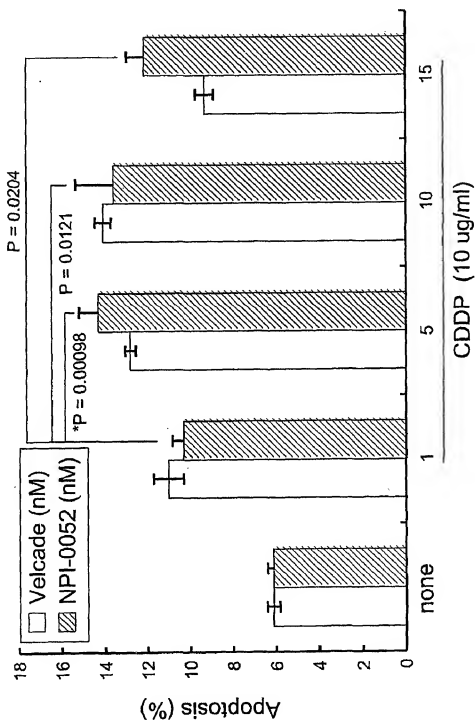


Fig. 4b

Combination between NPI-0052 and DHMEQ on
CDDP-induced apoptosis in Daudi RR1 cells

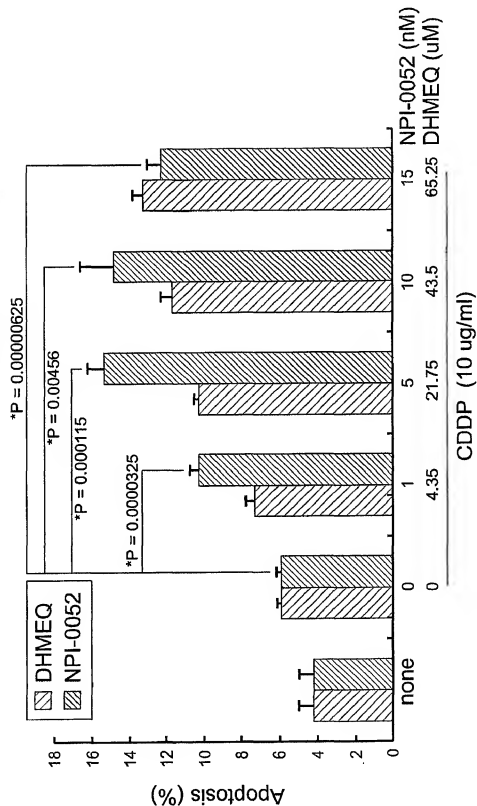


Fig. 5

Rituximab-mediated sensitization to NPI-induced apoptosis

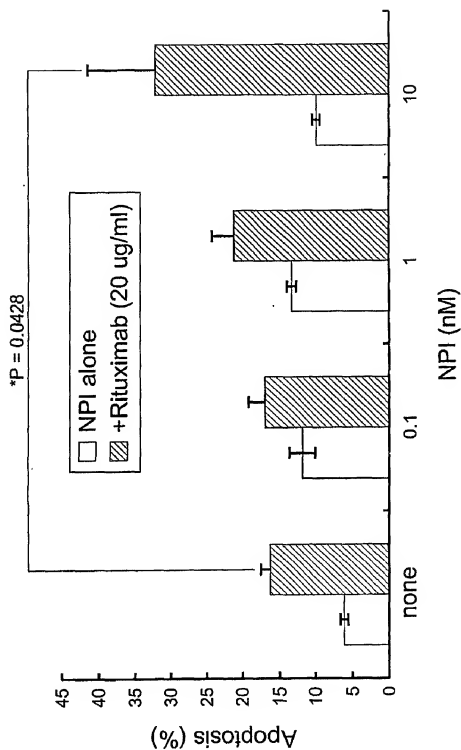


Fig. 6

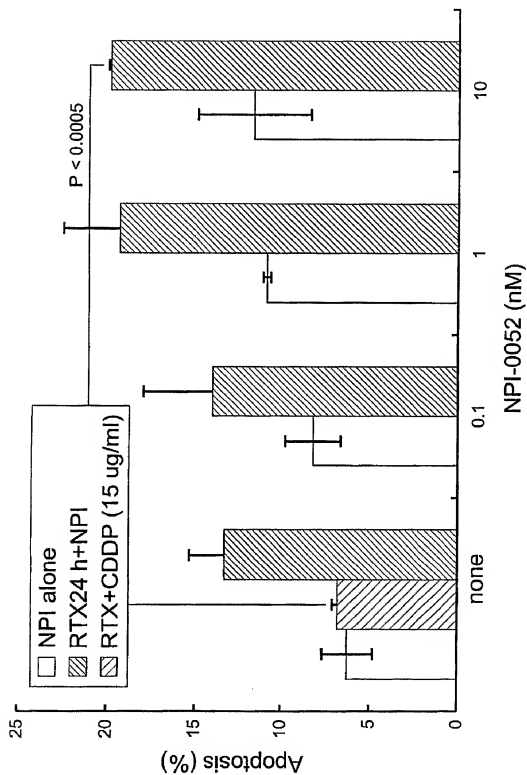
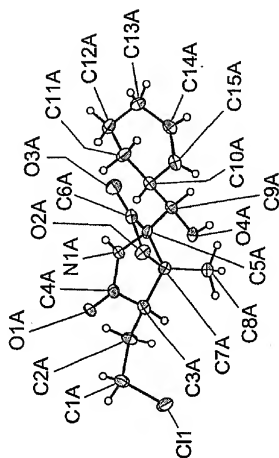
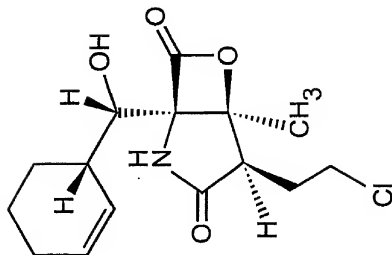


Fig. 7



ORTEP plot of the final X-ray
structure of NPI-0052 depicting
the absolute stereochemistry

*Fig. 7 (sheet 2)***Description of Compound**

- a. Nereus Identification Number: NPI-0052 (Salinosporamide A)
Small molecule, 20S proteasome inhibitor
- b. Pharmacological Class: Anti-cancer, Anti-inflammatory
- c. Chemical Class: Bicyclic γ -lactam β -lactone
- d. Molecular Formula: $C_{15}H_{20}ClNO_4$
- e. Molecular Weight: 313.78
- f. Chemical Structure: See Fig. 7 sheet 1

Form: White solid

Melting point: 169 – 171°C

UV Absorptivity (MeOH): λ_{\max} nm (log ϵ) 205 (4.03), 225 (3.3)

Optical Rotation $[\alpha]_D^{25}$: -72.9° (c = 0.55, MeOH)

Log D^{7.4}: 2.4 (See Section 7)

Solubility in PBS, pH 7.4: 9.6 μ M or 3 μ g/mL

Storage: Freeze solid at -20°C and store in the dark. Once reconstituted in DMSO, solutions are stable for several months at -80°C (20 mM stocks have been tested). NPI-0052 is also stable for at least three months as a solid.

Fig. 8

NPI-0052 Rituximab-mediated sensitization of
Ramos B-NHL cell line to NPI-induced apoptosis

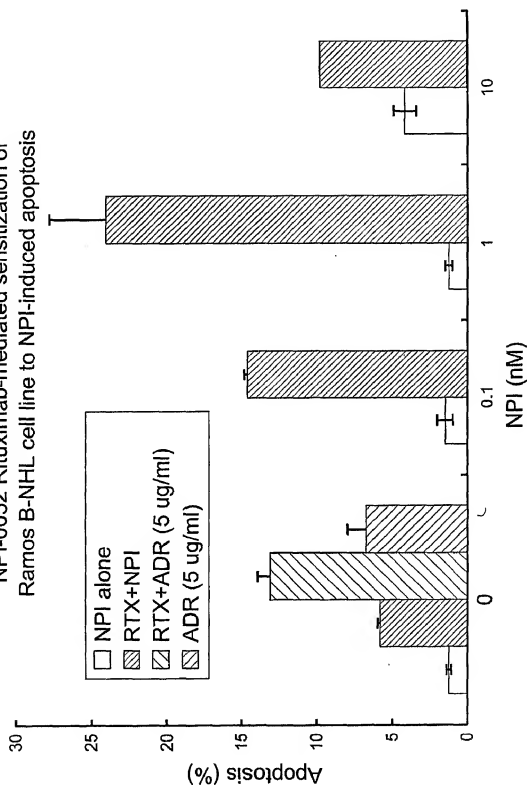


Fig. 9
NPI-0052 induces RKIP expression in Ramos

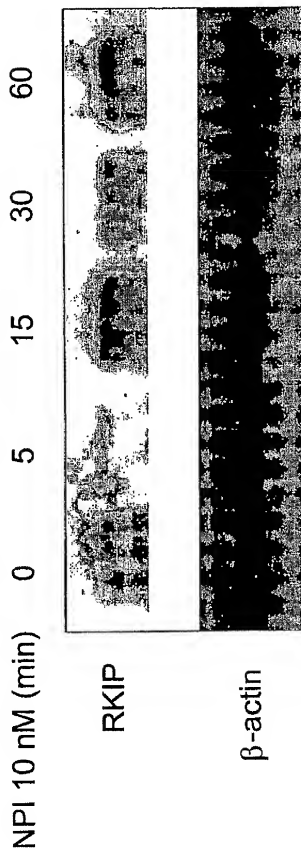


Fig. 10
NPI-0052 inhibits YY1 expression in Ramos

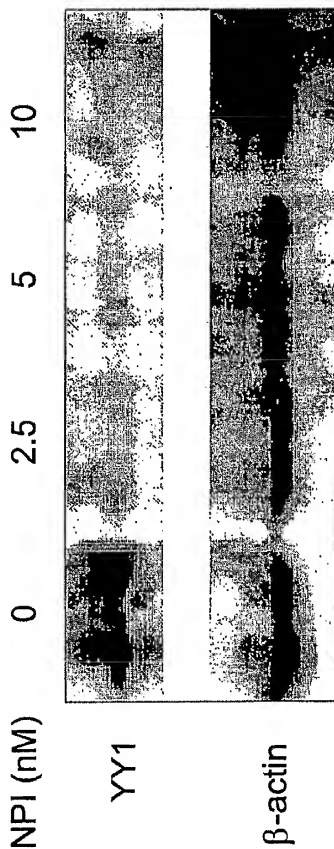


Fig. 11

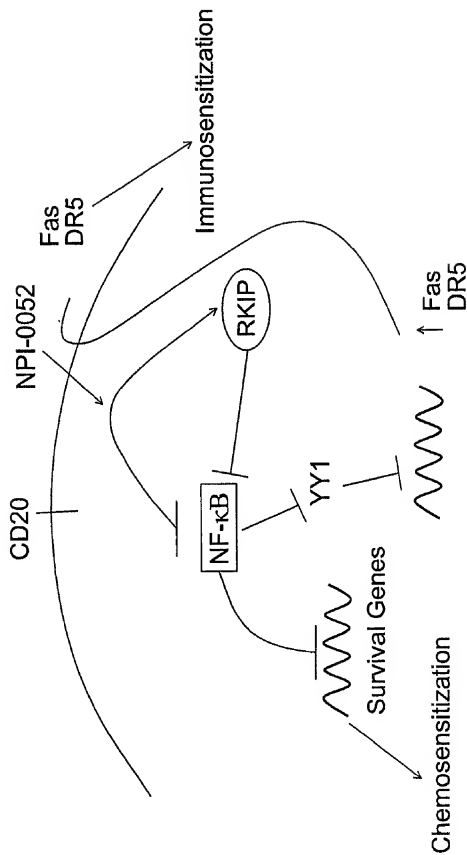


Fig. 12

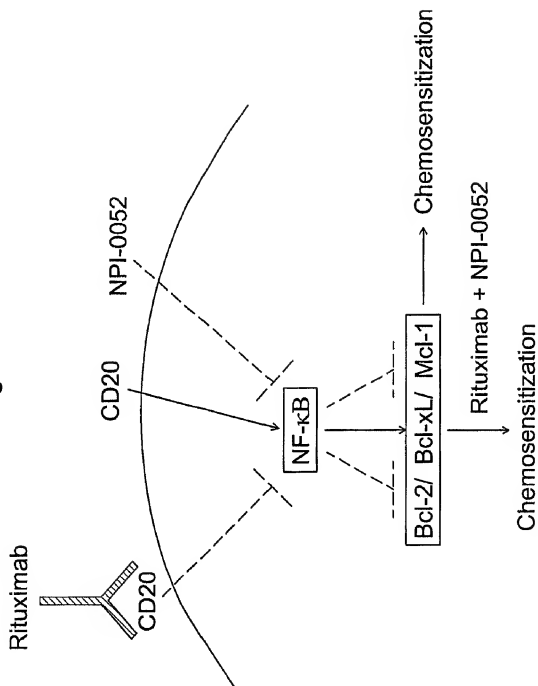
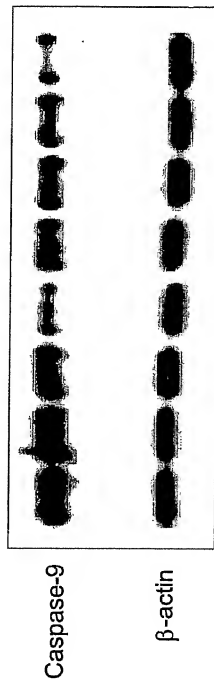


Fig. 13

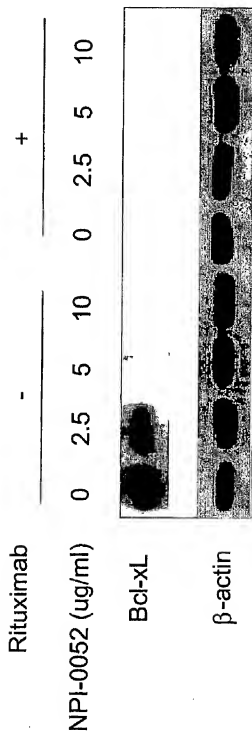
Combination treatment of NPI-0052 and
CDDP activated caspase-9



Ramos cells were treated with optimal concentration of NPI-0052 (2.5 nM) for 20 h, then treated with CDDP for an additional 24 hr. Total cell lysates were examined by WB for caspase-9 and β -actin expression.

Fig. 14

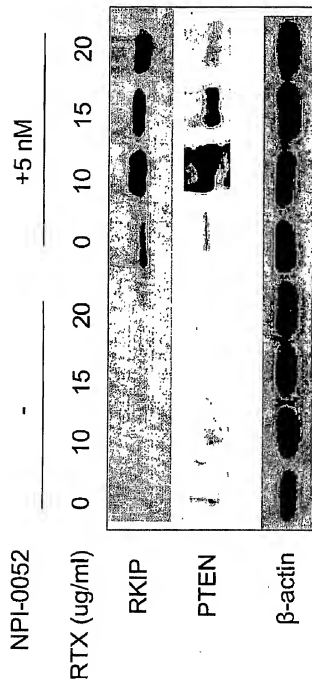
Both Rituximab and NPI inhibit Bcl-xL



Ramos cells were treated with optimal concentration of Rituximab (20 nM) for 20 h, then treated with NPI-0052 for an additional 24 hr. Total cell lysates were examined by WB for Bcl-xL and β -actin expression.

Fig. 15

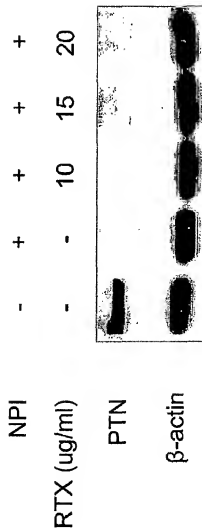
Combination treatment between NPI
and Rituximab induces RKIP and PTEN



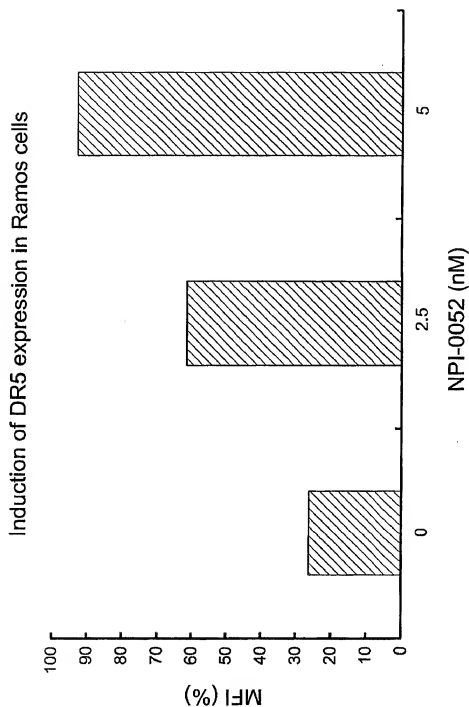
Ramos cells were treated with optimal concentration of NPI-0052 (5 nM) for 1 h, then treated with various concentrations of Rituximab for an additional 24 hr. Total cell lysates were examined by WB for RKIP, PTEN, and β -actin expression.

Fig. 16

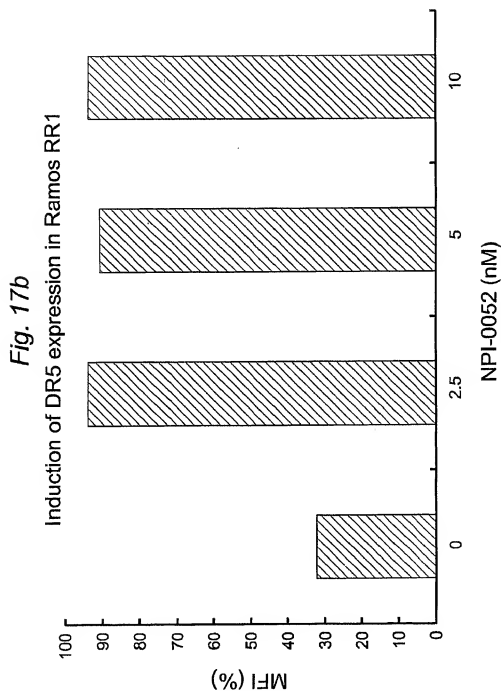
Combination treatment between NPI and Rituximab
results in inhibition of PTN (pleiotrophin)



Ramos cells were treated with optimal concentration of NPI-0052 (5 nM) for 1 h, then treated with various concentrations of Rituximab for an additional 24 hr. Total cell lysates were examined by WB for RKIP, PTEN, and I-actin expression.

Fig. 17a

Ramos cells were treated with various concentrations of NPI (0 - 5 nM) for 24 h, then DR5 expressions were observed by flow.



Ramos RR1 cells were treated with various concentrations of NPI (0 - 5 nM) for 24 h, then DR5 expressions were observed by flow.

Fig. 18

NPI sensitized Ramos cells to TRAIL-induced apoptosis

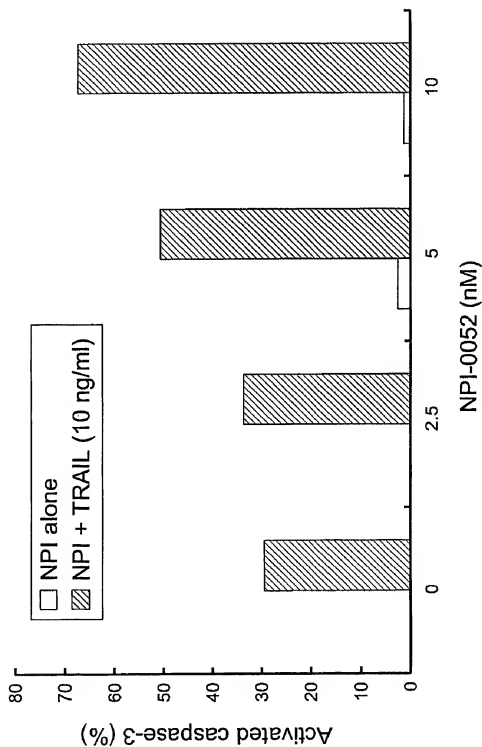
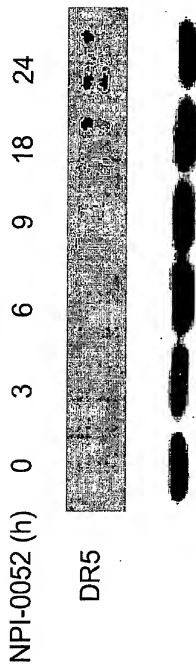


Fig. 19

Induction of DR5 expression in Ramos cells



Ramos cells were treated with NPI (2.5 nM) for various periods of time, then total cell lysates were prepared and examined by Western for DR5 and β -actin.

Fig. 20
NPI inhibited YY1 mRNA in Ramos cells

NPI (nM) 0 2.5 5 10



GAPDH

Ramos cells were treated with various concentrations of NPI (0 - 10 nM) for 24 h. Total RNAs were prepared and YY1 was examined by RT-PCR. GAPDH was loaded as control.

METHODS OF SENSITIZING CANCER TO THERAPY-INDUCED CYTOTOXICITY

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Ser. No. 60/733,965, filed on Nov. 4, 2005, and U.S. Ser. No. 60/840,811, filed Aug. 28, 2006, the teachings of which are incorporated herein by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] NOT APPLICABLE

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING
APPENDIX SUBMITTED ON A COMPACT DISK

[0003] NOT APPLICABLE

BACKGROUND OF THE INVENTION

[0004] Proteasome inhibitors have been shown to induce cell killing alone and/or in combination with drugs in drug-resistant tumor cells. In 2003, the FDA approved the first proteasome inhibitor VELCADE, "bortezomib" for treating patients with multiple myeloma who relapsed after two therapies and are progressing on current treatments. Thus, proteasome inhibitors prove to be clinically effective. However, like many other drugs, resistance to bortezomib starts to emerge as well as bortezomib-induced tissue toxicity has been noted. The development of new proteasome inhibitors that can override bortezomib resistance and exhibiting less toxicity is highly desirable. The chemical compound Salinosporamide A (NPI-0052, Nereus Pharmaceuticals, San Diego) was discovered during the fermentation of *Salinospora* species, a new marine gram positive actinomycete. It is related to two less potent 20S proteasome inhibitors, structurally related to lactacystin, omuralide, and PS-519.

[0005] Several in vitro findings indicated that Salinosporamide A exhibited cytotoxicity against a variety of tumor cell lines (Feling, et al., *Angew. Chem. Int. Ed.*, 2003, 42(3): 355-357) and can exert apoptosis and inhibition of NF- κ B (Machler, et al., *Journal of Medicinal Chemistry*, 2005, 48:3684). It has also been shown that Salinosporamide A is effective in bortezomib-resistant cell lines. In vivo, Salinosporamide A exerted anti-tumor effects whether administered orally or intravenously (Chuah et al., *Cancer Cell*, Nov. 8, 407-419 (2005)). Salinosporamide A has been synthesized chemically. Studies on cytotoxicity with the NCI screening panels of 60 human tumor cell lines showed that Salinosporamide A affected many cancer cells, and had a mean growth inhibition of less than 10 nM. Other tumor cell lines examined showed significant cytotoxic activity. Noteworthy, Salinosporamide A was also cytotoxic to both drug sensitive HL60 and drug resistant HL60MX2 with equal doses.

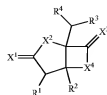
[0006] Salinosporamide A also had the effect of inducing a range of direct apoptosis on different tumor cell lines. The effect of Salinosporamide A on the induction of apoptosis suggests that Salinosporamide A may be used as an agent to identify anti-apoptotic pathways that may serve as targets for cancer therapy by examining changes in the expression of nucleic acids and proteins upon the treatment of cancer cells with this compound.

[0007] In the present application, we have examined if Salinosporamide A can sensitize therapy sensitive and therapy-resistant B Non-Hodgkin's Lymphoma to therapy-induced apoptosis. We also investigated whether Salinosporamide A could act as a therapeutic agent and induce apoptosis after sensitization by another compound such as rituximab. Furthermore, we have also examined the effect of Salinosporamide A on the induction of Raf kinase inhibitor protein (RKIP), a metastasis tumor suppressor protein that potentiates anti-apoptotic pathways in cancer cells, and on the inhibition of expression of YY1, a transcriptional regulator protein overexpressed in cancer cells that regulates tumor cell resistance to both chemotherapy and immunotherapy

BRIEF SUMMARY OF THE INVENTION

[0008] The present application demonstrates that Salinosporamide A, in combination with subtoxic therapeutically effective amounts of cancer therapeutic agents, sensitizes both resistant and sensitive cancer cells to therapy-induced cytotoxicity. The cancer cells can be either therapy-sensitive or therapy resistant. Furthermore, the present application demonstrates that Salinosporamide A acts as a therapeutic agent to induce apoptosis in cancer cells after sensitization of the cells by an antibody or by various chemo- and immunosensitizing agents. The cancer cells can be either therapy-sensitive or therapy resistant. Additionally, the present application demonstrates that Salinosporamide A induces the expression of RKIP, thereby inhibiting survival anti-apoptotic signaling pathways and resulting in reducing the threshold of anti-apoptotic gene expression and when used alone, or in combination with other agents, results in apoptosis. Furthermore, induction of RKIP also exerts anti-angiogenic activity as well as prevents metastasis. Further Salinosporamide A treatment inhibits the transcription repressor YY1, resulting in the upregulation of death receptors and sensitization of tumor cells to cytotoxic immunotherapy. It also regulates death receptor expression in rituximab-resistant clones. Salinosporamide A induces the expression of the AKT inhibitor PTEN resulting in downstream inhibition of the AKT anti-apoptotic and survival pathway and resulting in inhibition of anti-apoptotic gene products. Salinosporamide A also inhibits the overexpression of pleiotrophin (PTN) a growth factor and resistance factor in tumor cells and circulating levels of PTN have been shown to have a prognostic importance.

[0009] In a first embodiment, the invention provides a method of treating, preventing or inhibiting a cancer by administering to a subject a therapeutically effective amount of a cancer therapy reagent and a sensitizing effective amount of a compound of Formula I:



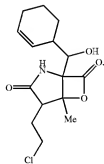
in which each of R¹, R² and R³ can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted

heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carbonyl, $-(C(O))H$, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfonyl; R^1 is a 5-8 membered cycloalkyl optionally substituted with 1-8 R^2 groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R^2 groups; each R^2 can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, or substituted cycloalkyl; each of X^1 , X^2 , X^3 and X^4 can be independently: O, NR^6 and S; and R^6 is H or C_1-C_6 alkyl.

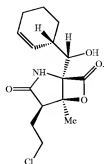
[0010] In some aspects of the first embodiment, each of R^1 and R^2 can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, and substituted cycloalkyl; R^3 can be alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfonyl; R^4 is a 5-8 membered cycloalkenyl optionally substituted with 1-8 R^5 groups; each of X^1 , X^2 and X^3 is O; and X^4 is NH.

[0011] In yet another aspect of the first embodiment, each of R^1 and R^2 can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, and substituted alkynyl; R^3 can be alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, and amino; R^4 is cyclohexenyl optionally substituted with 1-8 R^5 groups; each of X^1 , X^3 and X^4 is O; and X^2 is NH.

[0012] In further aspects of the first embodiment, R^1 is an alkyl or substituted alkyl; R^2 is alkyl; R^3 is hydroxy; R^4 is cyclohexenyl; and each of X^1 , X^3 and X^4 is O; and X^2 is NH; the substituted alkyl of R^1 can be a halogenated alkyl, which can be fluorinated, chlorinated, brominated in different aspects. In some aspects, the halogenated alkyl compound has the following structure:



[0013] In yet another aspect of the first embodiment, the halogenated alkyl compound has the following structure:



[0014] In further aspects of the first embodiment, the sensitizingly effective amount of the compound of Formula I is sufficient to induce expression of RKIP or PTEN, thereby inducing or facilitating apoptosis. The expression of RKIP or PTEN can be at least 1, 2, 10, or 100 fold higher than in the absence of the compound of Formula I.

[0015] In yet further aspects of the first embodiment, the sensitizingly effective amount of the compound of Formula I is sufficient to inhibit the expression of YY1, and PTEN, thereby inducing apoptosis. The expression of YY1, PTEN, and PTEN can be at least 1, 2, 10, or 100 fold lower than in the absence of the compound of Formula I.

[0016] In another aspect of the first embodiment, the cancer therapy reagent can be a chemotherapeutic reagent, an immunotherapeutic reagent, a radiotherapeutic reagent, a hormonal therapeutic reagent, or a pharmacologic inhibitor.

[0017] In other aspects of the first embodiment, the cancer can be non-Hodgkin's lymphoma, B-acute lymphoblastic lymphoma, prostate cancer, ovarian cancer, renal cancer, lung cancer, breast cancer, colon cancer, leukemia, multiple myeloma and hepatocarcinoma.

[0018] In another aspect of the first embodiment, the cancer therapy reagent induces or facilitates apoptosis and can be a chemotherapeutic reagent, an immunotherapeutic reagent, a radiotherapeutic reagent, a hormonal therapeutic reagent, or a pharmacologic inhibitor. In this aspect, the cancer therapy reagent can be rituximab immunotherapy.

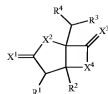
[0019] In various aspects of the first embodiment, the cancer is therapy-resistant, including resistance to immunotherapy, chemotherapy, radiotherapy, or hormonal therapy. However, in other aspects, the cancer can be therapy-sensitive.

[0020] In further aspects of the first embodiment, the therapeutically effective amount of a cancer therapy reagent and the sensitizingly effective amount of a compound of Formula I are administered concurrently or sequentially, in which the cancer therapy reagent is bortezomib administration. In related aspects, the cancer therapy reagent can be a chemotherapeutic reagent, an immunotherapeutic reagent, a radiotherapeutic reagent, a hormonal therapeutic reagent, or a pharmacologic inhibitor.

[0021] In an alternative aspect of the first embodiment, the therapeutically effective amount of a cancer therapy reagent and the sensitizingly effective amount of a compound of Formula I are administered sequentially.

[0022] In an aspect of the first embodiment, the subject can be a human.

[0023] A second embodiment of this invention provides a method of treating, preventing or inhibiting lymphoma by administering to a subject a therapeutically effective amount of a cancer therapy reagent and a sensitizingly effective amount of a compound of Formula I:



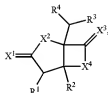
in which each of R^1 , R^2 and R^3 can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carbonyl, $-C(O)H$, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R^4 is a 5-8 membered cycloalkyl optionally substituted with 1-8 R^5 groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R^5 groups; each R^5 can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, and substituted cycloalkyl; each of X^1 , X^2 , X^3 and X^4 is independently selected from the group consisting of O, NR^6 and S; and R^6 is H or C_1 - C_6 alkyl.

[0024] In an aspect of the second embodiment, the sensitizingly effective amount of the compound of Formula I is sufficient to induce expression of RKIP or PTEN, thereby inducing or potentiating apoptosis.

[0025] In another aspect of the second embodiment, the sensitizingly effective amount of the compound of Formula I is sufficient to inhibit the expression of YY1 or PTN thereby inducing or potentiating apoptosis.

[0026] In further aspects of the second embodiment, the lymphoma is therapy resistant, which can include a lymphoma which is rituximab therapy resistant.

[0027] In a third embodiment, this invention provides a method of treating, preventing or inhibiting lymphoma by administering to a subject a therapeutically effective amount of rituximab and a sensitizingly effective amount of a compound of Formula I:



in which each of R^1 , R^2 and R^3 can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carbonyl, $-C(O)H$, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R^4 is a 5-8 membered cycloalkyl optionally substituted with 1-8 R^5 groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R^5 groups; each R^5 can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, and substituted cycloalkyl; each of X^1 , X^2 , X^3 and X^4 can independently be O, NR^6 and S; and R^6 is H or C_1 - C_6 alkyl.

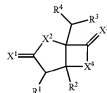
[0028] In an aspect of the third embodiment, the sensitizingly effective amount of the compound of Formula I is sufficient to induce expression of RKIP or PTEN, thereby inducing apoptosis.

[0029] In another aspect of the third embodiment, the sensitizingly effective amount of the compound of Formula I is sufficient to inhibit expression of YY1 or PTN, thereby inducing apoptosis.

[0030] In a fourth embodiment, the invention provides a composition containing a therapeutically effective amount of rituximab and a sensitizingly effective amount of a compound of Formula I in a physiologically acceptable excipient.

[0031] In a fifth embodiment, the invention provides a kit comprising a therapeutically effective amount of rituximab and a sensitizingly effective amount of a compound of Formula I.

[0032] In a sixth embodiment, this invention provides a method of treating, preventing or inhibiting a cancer with proteasome inhibitor therapy by administering to a subject a sensitizingly effective amount of an antibody or chemosensitizing reagent and a therapeutically effective amount of a compound of Formula I:



in which each of R^1 , R^2 and R^3 can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carbonyl, $-C(O)H$, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R^4 is a 5-8 membered cycloalkyl optionally substituted with 1-8 R^5 groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R^5 groups; each R^5 can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, and substituted cycloalkyl; each of X^1 , X^2 , X^3 and X^4 can independently be O, NR^6 and S; and R^6 is H or C_1 - C_6 alkyl.

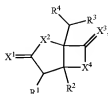
[0033] In an aspect of the sixth embodiment, the sensitizingly effective amount of the compound of Formula I is sufficient to induce expression of RKIP or PTEN, thereby inducing apoptosis or sensitizing cells to apoptosis by various sub-toxic concentrations on cytotoxic agents. In other aspects of this embodiment, the antibody is rituximab and the cancer is lymphoma.

[0034] In another aspect of the sixth embodiment, the sensitizingly effective amount of the compound of Formula I is sufficient to inhibit expression of YY1 or PTN, thereby inducing apoptosis. In other aspects of this embodiment, the antibody is rituximab and the cancer is lymphoma.

[0035] In a seventh embodiment, this invention provides a composition comprising a sensitizingly effective amount of rituximab and a therapeutically effective amount of a compound of Formula I in a physiologically acceptable excipient.

[0036] In an eighth embodiment, this invention provides a kit comprising a sensitizingly effective amount of rituximab and a therapeutically effective amount of a compound of Formula I.

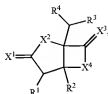
[0037] In a ninth embodiment, this invention provides a method of treating, preventing or inhibiting a cancer, the method comprising the step of administering to a subject a therapeutically effective amount of a compound of Formula I:



in which each of R¹, R² and R³ can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, —C(O)H, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R⁴ is a 5-8 membered cycloalkyl optionally substituted with 1-8 R⁵ groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups; each R⁵ can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, or substituted cycloalkyl; each of X¹, X², X³ and X⁴ can independently be O, NR⁶ and S; R⁶ is H or C₁-C₆ alkyl, and in which the therapeutically effective amount is sufficient to induce the expression of RKIP or PTEN, thereby inducing apoptosis.

[0038] In various aspects of the ninth embodiment, the expression of RKIP or PTEN is at least about 1, 2, 10, or 100 fold higher than in the absence of the compound of Formula I.

[0039] In a tenth embodiment, this invention provides a method of treating, preventing or inhibiting a cancer, the method comprising the step of administering to a subject a therapeutically effective amount of a compound of Formula I:

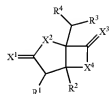


in which each of R¹, R² and R³ can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, —C(O)H, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R⁴ is a 5-8 membered cycloalkyl optionally substituted with 1-8 R⁵ groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups; each R⁵ can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, or substituted cycloalkyl; each of X¹, X², X³

and X⁴ can independently be O, NR⁶ and S; R⁶ is H or C₁-C₆ alkyl, and in which the therapeutically effective amount is sufficient to inhibit the expression of YY1 or PTEN, thereby inducing apoptosis or lowering the threshold of resistance to apoptosis by cytotoxic drugs.

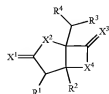
[0040] In various aspects of the tenth embodiment, the expression of YY1 or PTEN is at least about 1, 2, 10, or 100 fold lower than in the absence of the compound of Formula I.

[0041] In a eleventh embodiment, this invention provides a method of treating, preventing or inhibiting lymphoma by administering to a subject, optionally in combination with cytotoxic agents, a therapeutically effective amount of a compound of Formula I:



in which each of R¹, R² and R³ can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, —C(O)H, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R⁴ is a 5-8 membered cycloalkyl optionally substituted with 1-8 R⁵ groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups; each R⁵ can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, or substituted cycloalkyl; each of X¹, X², X³ and X⁴ can independently be O, NR⁶ and S; R⁶ is H or C₁-C₆ alkyl, and in which the therapeutically effective amount is sufficient to induce the expression of RKIP or PTEN, thereby inducing apoptosis.

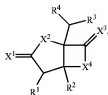
[0042] In a twelfth embodiment, this invention provides a method of treating, preventing or inhibiting lymphoma by administering to a subject a therapeutically effective amount of a compound of Formula I:



in which each of R¹, R² and R³ can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, —C(O)H, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R⁴ is a 5-8 membered cycloalkyl optionally

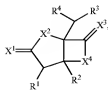
substituted with 1-8 R⁵ groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups; each R⁵ can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, or substituted cycloalkyl; each of X¹, X², X³ and X⁴ can independently be O, NR⁶ and S; R⁶ is H or C₁-C₆ alkyl, and in which the therapeutically effective amount is sufficient to inhibit the expression of YY1 or PTN, thereby inducing apoptosis.

[0043] In an thirteenth embodiment, this invention provides a method of treating, preventing or inhibiting a cancer with proteasome inhibitor therapy by administering to a subject a therapeutically effective amount of a compound of Formula I:



in which each of R¹, R² and R³ can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, —C(O)H, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R⁴ is a 5-8 membered cycloalkyl optionally substituted with 1-8 R⁵ groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups; each R⁵ can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, or substituted cycloalkyl; each of X¹, X², X³ and X⁴ is independently selected from the group consisting of O, NR⁶ and S; and R⁶ is H or C₁-C₆ alkyl, in which the therapeutically effective amount is sufficient to induce the expression of RKIP or PTEN, thereby inducing apoptosis.

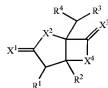
[0044] In an fourteenth embodiment, this invention provides a method of treating, preventing or inhibiting a cancer with proteasome inhibitor therapy by administering to a subject a therapeutically effective amount of a compound of Formula I:



in which each of R¹, R² and R³ can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, car-

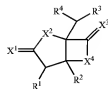
boxyl, —C(O)H, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R⁴ is a 5-8 membered cycloalkyl optionally substituted with 1-8 R⁵ groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups; each R⁵ can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, or substituted cycloalkyl; each of X¹, X², X³ and X⁴ is independently selected from the group consisting of O, NR⁶ and S; and R⁶ is H or C₁-C₆ alkyl, in which the therapeutically effective amount is sufficient to induce the expression of YY1 or PTN thereby inducing apoptosis.

[0045] In a fifteenth embodiment of this invention, this invention provides a method of treating a therapy resistant cancer by administering to a subject a therapeutically effective amount of a compound of Formula I:



in which each of R¹, R² and R³ can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, —C(O)H, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, or sulfuryl; R⁴ is a 5-8 membered cycloalkyl optionally substituted with 1-8 R⁵ groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups; each R⁵ can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, or substituted cycloalkyl; each of X¹, X², X³ and X⁴ can independently be O, NR⁶ and S; R⁶ is H or C₁-C₆ alkyl, in which the therapeutically effective amount is sufficient to induce the expression of RKIP or PTEN, thereby inducing apoptosis.

[0046] In a sixteenth embodiment of this invention, this invention provides a method of treating a therapy resistant cancer by administering to a subject a therapeutically effective amount of a compound of Formula I:



in which each of R¹, R² and R³ can independently be: H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, —C(O)H, acyl, oxyacyl, carbamate, sulfonyl, sulfona-

amide, or sulfonyl; R^4 is a 5-8 membered cycloalkyl optionally substituted with 1-8 R^2 groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R^2 groups; each R^2 can independently be: alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, or substituted cycloalkyl; each of X^1 , X^2 , X^3 and X^4 can independently be O, NR⁶ and S; R^6 is H or C₁-C₆ alkyl, in which the therapeutically effective amount is sufficient to inhibit the expression of YY1 or PTN, thereby inducing apoptosis.

BRIEF DESCRIPTION OF THE DRAWINGS

[0047] FIG. 1 shows that Salinosporamide A significantly sensitizes B-NHL Ramos cell line to CDDP-induced apoptosis.

[0048] Drug and Salinosporamide A resistant Ramos cells (106 ml) were treated with various concentrations of Salinosporamide A for one hour and then treated with CDDP (15 μ g/ml) for an additional 20 h. The cultures were then washed and the cells harvested and examined for apoptosis using the propidium iodide method, which measures DNA fragmentation, by flow cytometry. The percent apoptotic cells was recorded. Very long concentrations of Salinosporamide A (0.1 nM) were effective. The treatment were performed in duplicates.

[0049] FIG. 2A shows that Salinosporamide A sensitizes drug and Salinosporamide A resistant B-NHL Daudi cell line to CDDP-induced apoptosis. Daudi cells were treated for Ramos as in FIG. 1 above.

[0050] FIG. 2B shows that Salinosporamide A sensitizes B-NHL Rituximab resistant Daudi RR1 cell line to CDDP-induced apoptosis. The rituximab resistant clone Daudi-RR1 was treated as in FIG. 1 above.

[0051] FIG. 3A compares Salinosporamide A and bortezomib-induced sensitization of B-NHL Daudi WT cells to CDDP-induced apoptosis. Daudi cells were treated with various concentrations of bortezomib or Salinosporamide A for 1 h and then treated with CDDP (10 μ g/ml) for an additional 20 h and the cells were treated for apoptosis as described in FIG. 1 above.

[0052] FIG. 3B compares Salinosporamide A and DHMEQ-induced sensitization of B-NHL Daudi WT cells to CDDP-induced apoptosis. Daudi cells were treated with various concentrations of DHMEQ (μ M) and/or Salinosporamide A (nM) for 1 h and then treated with CDDP (10 μ g/ml) for an additional 20 h. The cells were then harvested and tested for apoptosis as described in FIG. 1 above.

[0053] FIG. 4A compares the effect of Salinosporamide A and bortezomib on CDDP-induced apoptosis in Daudi RR1 cells. The rituximab resistant Daudi RR1 clone was treated with various concentrations of bortezomib and/or Salinosporamide A for 1 h and then treated with CDDP 10 μ g/ml for an additional 20 h. These cells were then examined for apoptosis as described in FIG. 1 above.

[0054] FIG. 4B compares the effect of Salinosporamide A and DHMEQ on CDDP-induced apoptosis in Daudi RR1 cells. The rituximab resistant Daudi RR1 cells were treated with various concentrations of DHMEQ (μ M) or Salinosporamide A (nM) for 1 h and then treated with CDDP (10 μ g/ml) for an additional 20 h. The cells were then examined for apoptosis as described in FIG. 1 above.

[0055] FIG. 5 shows rituximab-mediated sensitization to Salinosporamide A-induced apoptosis. Ramos cells were treated with rituximab (20 μ g/ml) for 1 h and then treated with

various concentrations of Salinosporamide A for an additional 20 h. The cells were then examined for apoptosis as described above in FIG. 1.

[0056] FIG. 6 shows that in comparison to rituximab-mediated chemosensitization to CDDP, rituximab sensitizes to Salinosporamide A-induced apoptosis to a higher level than CDDP.

[0057] FIG. 7 shows the structure of Salinosporamide A.

[0058] FIG. 8 shows that rituximab sensitizes Ramos B-NHL cells to Salinosporamide A-induced apoptosis. Ramos cells were cultured untreated or treated with rituximab (20 μ g/ml) for 18 h. Thereafter, the cells were treated with adriamycin (ADR) (5 μ g/ml) or with different concentrations of Salinosporamide A (0.1, 1.0, 10 nM) overnight. The cell lines were examined for apoptosis by flow cytometry using the propidium iodide method for measuring DNA fragmentation.

[0059] FIG. 9 shows that treatment of tumor cells with Salinosporamide A results in the induction of the tumor suppressor, Raf-kinase inhibitor protein (RKIP). Ramos cells were treated with 10 nM Salinosporamide A for various periods of time, and total cell lysates were examined for the expression of RKIP by western blot analysis. The expression of β -actin was used as a control.

[0060] FIG. 10 shows that treatment of tumor cells with Salinosporamide A inhibits YY1 expression in Ramos B-NHL cells. Ramos cells were treated with various concentrations of Salinosporamide A for 24 hours, and total cell lysates were examined for the expression of YY1 by western blot analysis. The expression of β -actin was used as a control.

[0061] FIG. 11 shows a schematic diagram representing the effect of Salinosporamide A on sensitization of drug-resistant tumor cells to various drug-immune-induced apoptosis. Tumor cells constitutively express activated NF- κ B, which in turn regulates the transcription of various survival genes and anti-apoptotic genes as well as regulating the expression of the transcriptional repressor YY1. Treatment of the cells with Salinosporamide A results in inhibition of NF- κ B activity leading to inhibition of survival gene products and anti-apoptotic gene products and resulting in chemosensitization. In addition, inhibition of NF- κ B by Salinosporamide A also inhibits YY1, which we have shown to negatively regulate Fas and DR5 transcription. The inhibition of YY1 results in upregulation of Fas and DR5 expression and sensitizes cells to FasL and TRAIL-induced apoptosis.

[0062] FIG. 12 shows a schematic diagram representing tumor cells that express constitutively activated NF- κ B, which regulates several anti-apoptotic gene products such as Bcl-2, Bcl-xL, and Mcl-1. Previous studies have demonstrated that those anti-apoptotic gene products are important for maintaining tumor cell resistance to various chemotherapeutic drugs. We have shown that treatment with rituximab inhibits NF- κ B activity and also inhibits the above anti-apoptotic gene products and sensitizes the tumor cells to various chemotherapeutic drugs. Salinosporamide A also inhibits NF- κ B activity, and the combination of rituximab and Salinosporamide A results in complementation or synergy and apoptosis. The apoptosis is the result of rituximab-induced sensitization to Salinosporamide A apoptosis.

[0063] FIG. 13 demonstrates that Salinosporamide A activates caspase 9 in Ramos cells and in combination with CDDP more caspase 9 was activated. The activation of caspase 9 was assessed by western as the level of pro-caspase 9 was reduced following treatment.

[0064] FIG. 14 shows that Salinosporamide A inhibits the anti-apoptotic gene product Bcl-xL following treatment with very low concentrations (<2.5 nM). Bcl-xL expression was assessed by western.

[0065] FIG. 15 shows that Salinosporamide A induces the expression of RKIP and PTEN and in combination with rituximab more was expressed as assessed by western.

[0066] FIG. 16 shows that Salinosporamide A inhibits the growth factor pleiotrophin (PTN) expression significantly at the concentration of 5 nM.

[0067] FIG. 17A shows that Salinosporamide A treatment of Ramos upregulated DR5 surface expression as detected by flow cytometry.

[0068] FIG. 17B shows that Salinosporamide A upregulates DR5 expression in the rituximab resistant Ramos cells RR1.

[0069] FIG. 18 shows that Salinosporamide A sensitizes TRAIL-resistant Ramos cells to TRAIL-induced apoptosis.

[0070] FIG. 19 shows that Salinosporamide A upregulates DR5 expression in Ramos cells as determined by Western.

[0071] FIG. 20 shows that Salinosporamide A inhibits YY1 transcription as determined by RT-PCR.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0072] "Cancer" refers to human cancers and carcinomas, sarcomas, adenocarcinomas, lymphomas, leukemias, etc., including solid and lymphoid cancers, kidney, breast, lung, bladder, colon, ovarian, prostate, pancreas, stomach, brain, head and neck, skin, uterine, testicular, glioma, esophagus, and liver cancer, including hepatocarcinoma, lymphoma, including B-acute lymphoblastic lymphoma, non-Hodgkin's lymphomas (e.g., Burkitt's, Small Cell, and Large Cell lymphomas) and Hodgkin's lymphoma, leukemia (including AML, ALL, and CML), multiple myeloma, mantle cell lymphoma, Waldenstrom's macroglobulinemia, and Philadelphia positive cancers.

[0073] "Therapy resistant" cancers, tumor cells, and tumors refers to cancers that have become resistant to both apoptosis-mediated (e.g., through death receptor cell signaling, for example, Fas ligand receptor, TRAIL receptors, TNF-R1), various conventionally used chemotherapeutic drugs, hormonal drugs, and radiation, and non-apoptosis mediated (e.g., antimetabolites, anti-angiogenic, etc.) cancer therapies. "Therapy sensitive" cancers are not resistant to therapy. One of skill in the art will appreciate that some cancers are therapy sensitive to particular agents but not to others. Cancer therapies include chemotherapy, hormonal therapy, radiotherapy, immunotherapy, and gene therapy.

[0074] "Therapy-mediated or induced cytotoxicity" refers to all mechanisms by which cancer therapies kill or inhibit cancer cells, including but not limited to inhibition of proliferation, inhibition of angiogenesis, and cell death due to, for example, activation of apoptosis pathways (e.g., death receptor cell signaling, for example, Fas ligand receptor, TRAIL receptors, TNF-R1). Cancer therapies include chemotherapy, immunotherapy, radiotherapy, and hormonal therapy.

[0075] "Therapeutic treatment" and "cancer therapies" and "cancer therapy reagents" refers to apoptosis-mediated and non-apoptosis mediated cancer therapies that treat, prevent, or inhibit cancers, including chemotherapy, hormonal therapy (e.g., androgens, estrogens, antiestrogens (tamoxifen), progestins, thyroid hormones and adrenal cortical com-

pounds), radiotherapy, and immunotherapy (e.g., ZEVALIN, BEXXAR, RITUXAN (rituximab), HERCEPTIN). Cancer therapies can be enhanced by administration with a sensitizing agent, as described herein, either before or with the cancer therapy.

[0076] "Chemotherapeutic drugs" include conventional chemotherapeutic reagents such as alkylating agents, antimetabolites, plant alkaloids, antibiotics, and miscellaneous compounds e.g., cis-platinum, CDDP, methotrexate, vincristine, adriamycin, bleomycin, and hydroxyurea. Chemotherapeutic drugs also include proteasome inhibitors such as salinosporamides (e.g., Salinosporamide A), bortezomib, PS-519, omuralide, PR-171 and its analogs, and Gleeevec. The drugs can be administered alone or in combination ("combination chemotherapy").

[0077] By "sensitizingly effective amount or dose" or "sensitizingly sufficient amount or dose" herein is meant a dose that produces cancer cell sensitizing effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (see, e.g., Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); Pickar, *Dosage Calculations* (1999); and Remington: *The Science and Practice of Pharmacy*, 20th Edition, 2003, Gennaro, Ed., Lippincott, Williams & Wilkins). Sensitized cancer cells respond better to cancer therapy (are inhibited or killed faster or more often) than non-sensitized cells, as follows: Control samples (untreated with sensitizing agents) are assigned a relative cancer therapy response value of 100%. Sensitization is achieved when the cancer therapy response value relative to the control is about 110% or 120%, preferably 200%, more preferably 500-1000% or more, i.e., at least about 10% more cells are killed or inhibited, or the cells are killed or inhibited at least about 10% faster. Cancer therapy response value refers to the amount of killing or inhibition of a cancer cell, or the speed of killing or inhibition of a cancer cell when it is treated with a cancer therapy. Some compounds are useful both as therapeutic reagents and as sensitizing reagents. Often, a lower dose (i.e., lower than the conventional therapeutic dose) or sub-toxic dose of such a reagent can be used to sensitize a cell. Often, when a cell is sensitized, a lower dose of the chemotherapeutic reagent can be used to achieve the same therapeutic effect as with a cell that has not been sensitized.

[0078] By "therapeutically effective amount or dose" or "therapeutically sufficient amount or dose" herein is meant a dose that produces therapeutic effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (see, e.g., Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); Pickar, *Dosage Calculations* (1999); and Remington: *The Science and Practice of Pharmacy*, 20th Edition, 2003, Gennaro, Ed., Lippincott, Williams & Wilkins). In sensitized cells, the therapeutically effective dose can often be lower than the conventional therapeutically effective dose for non-sensitized cells.

[0079] Apoptosis refers to a process of programmed cell death that is different from the general cell death or necrosis that results from exposure of cells to non-specific toxic events such as metabolic poisons or ischemia in being an ordered molecular process by which unwanted cells undergo death.

Cells undergoing apoptosis show characteristic morphological changes such as chromatin condensation and fragmentation and breakdown of the nuclear envelope in a process called pyknosis. As apoptosis proceeds, the plasma membrane is seen to form blebs and the apoptotic cells are either phagocytosed or else break up into smaller vesicles which are then phagocytosed. Typical assays used to detect and measure apoptosis include microscopic examination of pyknotic bodies as well as enzymatic assays such as TUNEL labeling, caspase assay, annexin assay, and DNA laddering, among others. Apoptotic cells can be quantitated by FACS analysis of cells stained with propidium iodide for DNA hypodiploidy.

[0080] "Inducing apoptosis" refers to an agent or process which causes a cell to undergo the program of cell death described above for apoptosis.

[0081] "Salinosporamide" refers to proteasome inhibitor compounds produced by *Salinospora* sp., a marine gram positive actinomycete, e.g., Salinosporamide A (Salinosporamide A), B, C, etc, and analogs thereof. Salinosporamides can be made by isolating the products from fermentation of *Salinospora* (wild type and mutant strains) and genetically engineered microorganisms, by biosynthesis in vitro using whole cells, enzymes, and recombinant enzymes, and by synthetic chemistry techniques.

[0082] "Salinosporamide A" refers to proteasome inhibitor compounds produced by *Salinospora* sp., a marine gram positive actinomycete. This term also refers to analogs of Salinosporamide A. Salinosporamide A and analogs thereof have structures as disclosed herein, e.g., in Formula 1 and FIG. 7, as well as in US20050049294, herein incorporated by reference.

[0083] "Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding.

[0084] Antibodies exist, e.g., as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab')_2$, a dimer of Fab which itself is a light chain joined to V_H - C_H1 by a disulfide bond. The $F(ab')_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the $F(ab')_2$ dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (see *Fundamental Immunology* (Paul et al., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized de novo using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (see, e.g., McCafferty et al., *Nature* 348:552-554 (1990)).

[0085] For preparation of antibodies, e.g., recombinant, monoclonal, or polyclonal antibodies, many techniques known in the art can be used (see, e.g., Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor et al., *Immunology Today* 4: 72 (1983); Cole et al., pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985); Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies, A Laboratory Manual* (1988); and Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986)). The genes encoding the heavy and light chains of an antibody of interest can be cloned from a cell, e.g., the genes encoding a monoclonal antibody can be cloned from a hybridoma and used to produce a recombinant monoclonal antibody. Gene libraries encoding heavy and light chains of monoclonal antibodies can also be made from hybridoma or plasma cells. Random combinations of the heavy and light chain gene products generate a large pool of antibodies with different antigenic specificity (see, e.g., Kuby, *Immunology* (3rd ed. 1997)). Techniques for the production of single chain antibodies or recombinant antibodies (U.S. Pat. No. 4,946, 778, U.S. Pat. No. 4,816,567) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized or human antibodies (see, e.g., U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, Marks et al., *BioTechnology* 10:779-783 (1992); Lonberg et al., *Nature* 368:856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14:845-51 (1996); Neuberger, *Nature Biotechnology* 14:826 (1996); and Lonberg & Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995)). Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (see, e.g., McCafferty et al., *Nature* 348:552-554 (1990); Marks et al., *Biotechnology* 10:779-783 (1992)). Antibodies can also be made bispecific, i.e., able to recognize two different antigens (see, e.g., WO 93/08829, Trautnecker et al., *EMBO J.* 10:3655-3659 (1991); and Suresh et al., *Methods in Enzymology* 121:210 (1986)). Antibodies can also be heteroconjugates, e.g., two covalently joined antibodies, or immunotoxins (see, e.g., U.S. Pat. No. 4,676,980, WO 91/00360; WO 92/200373; and EP 03089).

[0086] Methods for humanizing or primatizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers (see, e.g., Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332: 323-327 (1988); Verhoeven et al., *Science* 239:1534-1536 (1988) and Presta, *Curr. Opin. Struct. Biol.* 2:593-596 (1992)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0087] A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

[0088] As used herein, the term "alkyl" refers to a monovalent straight or branched chain hydrocarbon group having from one to about 12 carbon atoms, including methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, tert-butyl, pentyl, hexyl, and the like.

[0089] As used herein, the term "substituted alkyl" refers to alkyl groups further bearing one or more substituents selected from hydroxy, alkoxy, mercapto, cycloalkyl, substituted cycloalkyl, heterocyclic, substituted heterocyclic, aryl, substituted aryl, heteroaryl, substituted heteroaryl, aryloxy, substituted aryloxy, halogen, cyano, nitro, amino, amido, $-C(O)H$, acyl, oxyacyl, carboxyl, sulfonyl, sulfonamide, sulfonyl, and the like.

[0090] As used herein, the term "lower alkyl" refers to alkyl groups having from 1 to about 6 carbon atoms.

[0091] As used herein, the term "alkenyl" refers to straight or branched chain hydrocarbyl groups having one or more carbon-carbon double bonds, and having in the range of about 2 up to 12 carbon atoms, and "substituted alkenyl" refers to alkenyl groups further bearing one or more substituents as set forth above. Alkenyl groups useful in the present invention include, but are not limited to, ethenyl, n-propenyl, isopropenyl, n-butenyl, isobutenyl, pentenyl, hexenyl, and the like.

[0092] As used herein, the term "alkynyl" refers to straight or branched chain hydrocarbyl groups having at least one carbon-carbon triple bond, and having in the range of about 2 up to 12 carbon atoms, and "substituted alkynyl" refers to alkynyl groups further bearing one or more substituents as set forth above. Alkynyl groups useful in the present invention include, but are not limited to, ethynyl, propynyl, butynyl, pentynyl, hexynyl, and the like.

[0093] As used herein, the term "aryl" refers to aromatic groups having in the range of 6 up to 14 carbon atoms and 1 to 3 rings, and "substituted aryl" refers to aryl groups further bearing one or more substituents as set forth above. Aryl groups useful in the present invention include, but are not limited to, phenyl, benzyl, naphthyl, biphenyl, phenanthrenyl, and anthrenyl.

[0094] As used herein, the term "heteroaryl" refers to aromatic rings containing one or more heteroatoms (e.g., N, O, S, or the like) as part of the ring structure, having in the range of 3 up to 14 carbon atoms and 1 to 3 rings. "Substituted heteroaryl" refers to heteroaryl groups further bearing one or more substituents as set forth above. Heteroaryl groups useful in the present invention include, but are not limited to, pyridyl, pyridyl N-oxide, indolyl, indazolyl, quinoxalyl, quinolyl, isoquinolyl, benzothienyl, benzofuranyl, benzopyranyl, benzothiopyranyl, furanyl, pyrrolyl, thiazolyl, benzothiazolyl, oxazolyl, isoxazolyl, triazolyl, tetrazolyl, pyrazolyl, imidazolyl, and thienyl.

[0095] As used herein, the term "alkoxy" refers to the moiety $-O-alkyl-$, wherein alkyl is as defined above, and "substituted alkoxy" refers to alkoxy groups further bearing one or more substituents as set forth above.

[0096] As used herein, the term "thioalkyl" refers to the moiety $-S-alkyl-$, wherein alkyl is as defined above, and "substituted thioalkyl" refers to thioalkyl groups further bearing one or more substituents as set forth above.

[0097] As used herein, the term "cycloalkyl" refers to ring-containing alkyl groups containing in the range of about 3 up to 8 carbon atoms, and "substituted cycloalkyl" refers to cycloalkyl groups further bearing one or more substituents as set forth above. Cycloalkyl groups useful in the present invention include, but are not limited to, cyclopropane, cyclobutane, cyclopentane, cyclohexane, cycloheptane and cyclooctane.

[0098] As used herein, the term "cycloalkenyl" refers to a 3 to 8 membered cycloalkyl group having at least one carbon-carbon double bond (alkene) in the ring, and "substituted cycloalkenyl" refers to cycloalkenyl groups further bearing one or more substituents as set forth above. Cycloalkenyl rings useful in the present invention include, but are not limited to, 1-cyclopentenyl, 2-cyclopentenyl, 3-cyclopentenyl, 1-cyclohexenyl, 2-cyclohexenyl, 3-cyclohexenyl, as well as cyclopropenyl, cyclobutenyl, cyclopentenyl and cyclooctenyl. Cycloalkadienyls are also useful in the present invention and include, but are not limited to, cyclopentadienyl, cyclohexadienyl, cycloheptadienyl and cyclooctadienyl.

[0099] As used herein, the term "heterocyclic" refers to cyclic (i.e., ring-containing) groups containing one or more heteroatoms (e.g., N, O, S, or the like) as part of the ring structure, having in the range of 3 up to 14 carbon atoms and 1 to 3 rings. "Substituted heterocyclic" refers to heterocyclic groups further bearing one or more substituents as set forth above. Heterocyclic groups useful in the present invention include, but are not limited to, pyrrolidinyl, pyrrolinyl, imidazolyl, pyrazolidinyl, pyrazolyl, piperidinyl, piperazinyl, indolyl, quinuclidinyl, morpholinyl, tetrahydrofuranly, tetrahydrothienyl and dioxane.

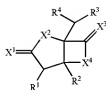
[0100] The compounds of the invention may be formulated into pharmaceutical compositions as natural or salt forms. Pharmaceutically acceptable non-toxic salts include the base addition salts (formed with free carboxyl or other anionic groups) which may be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, and ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino-ethanol, histidine, procaine, and the like. Such salts may also be formed as acid addition salts with any free cationic groups and will generally be formed with inorganic acids such as, for example, hydrochloric, sulfuric, or phosphoric acids, or organic acids such as acetic, p-toluenesulfonic, methanesulfonic acid, oxalic, tartaric, mandelic, and the like. Salts of the invention include amine salts formed by the protonation of an amino group with inorganic acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, phosphoric acid, and the like. Salts of the invention also include amine salts formed by the protonation of an amino group with suitable organic acids, such as p-toluenesulfonic acid, acetic acid, and the like. Additional excipients which are contemplated for use in the practice of the present invention are those available to those of ordinary skill in the art, for example, those found in the *United States Pharmacopoeia* Vol. XXII and *National Formulary* Vol. XVII, U.S. Pharmacopoeia Convention, Inc., Rockville, Md. (1989), the relevant contents of which is incorporated herein by reference.

[0101] The compounds according to this invention may contain one or more asymmetric carbon atoms and thus occur as racemates and racemic mixtures, single enantiomers, diastereomeric mixtures and individual diastereomers. The term "stereoisomer" refers to chemical compounds which differ from each other only in the way that the different groups in the molecules are oriented in space. Stereoisomers have the same molecular weight, chemical composition, and constitution as another, but with the atoms grouped differently. That is, certain identical chemical moieties are at different orientations in space and, therefore, when pure, have the ability to rotate the plane of polarized light. However, some pure stereoisomers may have an optical rotation that is so slight that it is undetectable with present instrumentation. All such isomeric forms of these compounds are expressly included in the present invention.

[0102] Each stereogenic carbon may be of R or S configuration. Although the specific compounds exemplified in this application may be depicted in a particular configuration, compounds having either the opposite stereochemistry at any given chiral center or mixtures thereof are also envisioned. When chiral centers are found in the derivatives of this invention, it is to be understood that this invention encompasses all possible stereoisomers. The terms "optically pure compound" or "optically pure isomer" refers to a single stereoisomer of a chiral compound regardless of the configuration of the compound.

II. Compounds

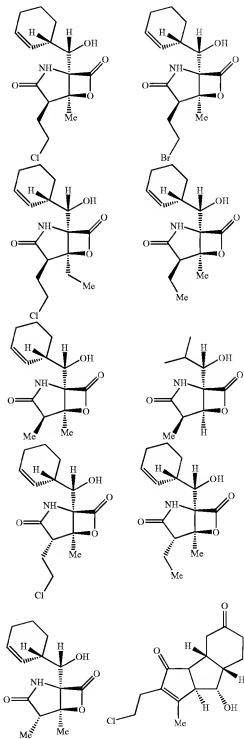
[0103] Compounds useful in the present invention include those of Formula I:



I

wherein each of R¹, R² and R³ are independently selected from the group consisting of H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, —C(O)H, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, and sulfuryl. R⁴ is a 5-8 membered cycloalkyl optionally substituted with 1-8 R⁵ groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups. Each R⁵ is independently selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, and substituted cycloalkyl. Each of X¹, X², X³ and X⁴ is independently selected from the group consisting of O, NR⁶ and S. And R⁶ is H or C₁-C₆ alkyl.

[0104] Additional compounds useful in the present invention include the following:



Salinosporamides disclosed in *J. Org. Chem.*, 70(16), 6196-6203, 2005 are incorporated herein by reference. Additional Salinosporamides are described in US20050049294, herein incorporated by reference in its entirety.

[10105] The compounds of the present invention can be prepared by a variety of methods including fermentation, recombinant biosynthesis and via synthetic methodologies.

[10106] A. Fermentation

[10107] The compounds of the present invention can be prepared, for example, by bacterial fermentation, which generates the compounds in sufficient amounts for pharmaceutical drug development and for clinical trials. In some embodiments, invention compounds are produced by fermentation of the actinomycete strains CN3392 and CN3476 in AIBIe-4 or CKA-liquid media. Essential trace elements which are necessary for the growth and development of the culture should also be included in the culture medium. Such trace elements commonly occur as impurities in other constituents of the medium in amounts sufficient to meet the growth requirements of the organisms. It may be desirable to add small amounts (i.e. 0.2 mL/L) of an antifoam agent such as polypropylene glycol (M.W. about 2000) to large scale cultivation media if foaming becomes a problem. The organic metabolites are isolated by adsorption onto an amberlite XAD-16 resin. For example, Salinosporamide A is isolated by elution of the XAD-16 resin with methanol:dichloromethane 1:1, which affords about 105 mg crude extract per liter of culture. Salinosporamide A is then isolated from the crude extract by reversed-phase flash chromatography followed by reverse-phase HPLC and normal phase HPLC, which yields 6.7 mg of Salinosporamide A. FIG. 5 and Example 1 of US 2004/0259856 (incorporated herein by reference) set forth a fermentation procedure for the preparation of the compounds of the instant invention. US20050049294, herein incorporated by reference in its entirety, also provides methods of isolating the compounds from fermentation broth.

[10108] B. Recombinant Biosynthesis

[10109] Recombinant biosynthesis uses cells expressing cloned genes and optionally naturally occurring pathways to create biosynthetic pathways to produce natural and novel metabolites (see, e.g., Albreuter et al., *Curr Opin Biotechnol*, 10:130-136 (1999); Reynolds, *PNAS* 95:12744-12746 (1998); and Cane et al., *Science* 282:63-68 (1998)). Several biosynthetic pathways are possible for the production of the compounds of the present invention, including a mixed polyketide-non-ribosomal peptide synthesis pathway. Polyketides and non-ribosomal peptides are synthesized from small chain carboxylic acid and amino acid monomers, respectively, by large multifunctional protein complexes called polyketide synthetases and nonribosomal peptide synthetases. US20050049294, herein incorporated by reference in its entirety, also provides information on recombinant biosynthesis.

[10110] C. Synthetic procedure

[10111] the compounds of the present invention can also be prepared using standard organic synthesis procedures known in the art. An exemplary synthetic procedure can be found in US 2005/0228186 (incorporated herein by reference) for the synthesis of

One of skill in the art will recognize that additional pathways exist for the synthetic preparation of the compounds of the present invention. US20050049294, herein incorporated by reference in its entirety, also provides information on synthesis of the compounds.

III. Methods

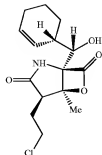
[10112] As described herein, Salinosporamide A is useful for sensitizing both sensitive and resistant cancer cells to therapy based apoptosis when administered in combination with low dose or sub-toxic amounts of cancer therapeutic reagents. Salinosporamide A and the low dose or sub-toxic amount of a cancer therapeutic can be administered alone to sensitize cells for subsequent therapies or co-administered in combination with chemotherapy, radiotherapy, hormonal therapy, or immunotherapy. In another embodiment, Salinosporamide A is used as a chemotherapeutic agent after cellular sensitization using an antibody. Salinosporamide A as a therapeutic can be administered alone or co-administered in combination with chemotherapy, radiotherapy, hormonal therapy, or immunotherapy. Methods of using Salinosporamide A are also described in US patent application 20050239866 and 20050049294, herein incorporated by reference in their entirety.

[10113] Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., *Remington's Pharmaceutical Sciences*, 20th ed., 2003, supra).

[10114] Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the compound suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, e.g., sucrose, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

[10115] The compound of choice, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[10116] Suitable formulations for rectal administration include, for example, suppositories, which consist of the compound with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the com-



pound of choice with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

[0117] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intratumoral, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration, oral administration, and intravenous administration are the preferred methods of administration. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

[0118] Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by nucleic acids for *ex vivo* therapy can also be administered intravenously or parenterally as described above.

[0119] The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into units containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packaged tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form. The composition can, if desired, also contain other compatible therapeutic agents.

[0120] Preferred pharmaceutical preparations deliver one or the compounds of the invention, optionally in combination with one or more therapeutic agents, in a sustained release formulation. Typically, Salinosporamide A is administered therapeutically as a sensitizing agent that increases the susceptibility of tumor cells to other cytotoxic cancer therapies, including chemotherapy, radiation therapy, immunotherapy and hormonal therapy. In some embodiments, Salinosporamide A acts as a chemotherapeutic reagent after cellular sensitization using an antibody.

[0121] In therapeutic use for the treatment of cancer, the compounds utilized in the pharmaceutical method of the invention are administered at the initial dosage of about 0.001 mg/kg to about 1000 mg/kg daily. A daily dose range of about 0.01 mg/kg to about 500 mg/kg, or about 0.1 mg/kg to about 200 mg/kg, or about 1 mg/kg to about 100 mg/kg, or about 10 mg/kg to about 50 mg/kg, can be used. The dosages, however, may be varied depending upon the requirements of the patient, the severity of the condition being treated, and the compound being employed. For example, dosages can be empirically determined considering the type and stage of cancer diagnosed in a particular patient. The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular compound in a particular patient. Determination of the proper

dosage for a particular situation is within the skill of the practitioner. Generally, treatment is initiated with smaller dosages which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day, if desired.

[0122] The pharmaceutical preparations are typically delivered to a mammal, including humans and non-human mammals. Non-human mammals treated using the present methods include domesticated animals (i.e., canine, feline, murine, rodentia, and lagomorpha) and agricultural animals (bovine, equine, ovine, porcine).

EXAMPLES

[0123] The following examples are offered to illustrate, but not to limit the claimed invention.

Example I

Salinosporamide A Induced Sensitization

1) Salinosporamide A-Induced Sensitization of Drug-Resistant B-NHL Ramos and Daudi Cell Lines to CDDP-Induced Apoptosis

[0124] The CDDP resistant B-NHL Ramos cell line was treated with various concentrations of Salinosporamide A for one hour and then treated with predetermined nontoxic concentration of CDDP (15 μ g/ml) for an additional 20 hours. The cells were then harvested and examined for apoptosis using the propidium iodide (PI) technique by flow cytometry examining DNA fragmentation. FIG. 1 shows that the combination treatment with Salinosporamide A and CDDP resulted in significant potentiation of cytotoxicity. In addition, Salinosporamide A treatment alone showed modest cytotoxicity at the concentration of 1 and 10 nM. The potentiation of cytotoxicity was mostly observed at very low concentrations of Salinosporamide A (0.1 nM) and significant synergistic cytotoxicity was observed. Similar studies were performed with the Daudi B-NHL cell line. Like Ramos, significant cytotoxicity was observed and the extent of cytotoxicity was a function of the concentration of Salinosporamide A used (FIG. 2A). These findings demonstrate that Salinosporamide A sensitizes both Ramos and Daudi B-NHL cells to CDDP-induced apoptosis.

2) Salinosporamide A-Mediated Sensitization of Rituximab Resistant Daudi Clone (Daudi RRI) to CDDP-Induced Apoptosis.

[0125] Rituximab (chimeric anti-CD20 monoclonal antibody) has been used in the treatment of Non-Hodgkin's lymphoma alone or in combination with chemotherapy. The clinical response has been very encouraging; however, some patients are initially unresponsive or develop resistance following treatment. In order to investigate the mechanism of rituximab resistance we have developed in our laboratory rituximab resistant clones from B-NHL cell lines. We have selected certain clones for further analysis of the underlying mechanism of resistance. In the present study we have examined the Daudi RRI clone which is resistant to rituximab-induced signaling and unlike Daudi wild type, rituximab failed to sensitize Daudi RRI to drug-induced apoptosis. In addition, we have found that Daudi RRI also develops the

highest degree of drug resistance compared to wild type. We investigated whether Salinosporamide A can sensitize Daudi RR1 to CDDP-induced apoptosis. FIG. 2B demonstrates that indeed Salinosporamide A significantly sensitized Daudi RR1 to CDDP-induced apoptosis and the extent of potentiation of cytotoxicity was a function of the concentration of Salinosporamide A used. These findings demonstrate that Salinosporamide A may be used clinically to reverse rituximab resistance to chemotherapy.

3) Comparison Between Salinosporamide A and Bortezomib in their Ability to Sensitize B-NHL Cell Lines to CDDP-Induced Apoptosis

[0126] a) Study with Daudi and Wild Type Cells

[0127] We examined the effect of Salinosporamide A and bortezomib in their ability to sensitize Daudi wild type cells to CDDP-induced apoptosis. The tumor cells were treated for one hour with various concentrations of Salinosporamide A or bortezomib (range 1-15 nM) and then treated with CDDP (10 µg/ml) for an additional 20 h. The cells were then examined for apoptosis as described above. The findings shown in FIG. 3A demonstrate that both agents yielded comparable results with respect to chemosensitization and with equivalent concentration dependent effects. There were significant apoptosis by the combination treatment at all concentrations of inhibitors used.

[0128] b) Study with Daudi RR1

[0129] We performed similar experiments as above with rituximab resistant Daudi RR1 cells and the findings are summarized in FIG. 4A. Like Daudi wild type, significant potentiation of apoptosis was observed by both agents. In the combination treatment with CDDP, both inhibitors showed similar patterns of potentiation of cytotoxicity.

[0130] The findings with both Daudi and Daudi RR1 cells demonstrate that Salinosporamide A and bortezomib showed similar findings under the conditions used and the model system utilized. Further analysis by changing the time of treatment and with other cell lines will determine if there were differences with respect to the concentrations and cytotoxicity when using Salinosporamide A or bortezomib in sensitization experiments.

4) Comparison between Salinosporamide A and the NF-κB inhibitor, DHMEQ in their ability to sensitize drug resistant tumor cells to CDDP-induced apoptosis.

[0131] a) Study with Daudi Wild Type Cells

[0132] DHMEQ is a NF-κB inhibitor that has been shown to be selective and preventing NF-κB translocation from the cytoplasm to the nucleus (Horiguchi, et al., Expert Rev. Anti-cancer Ther., 2003, 3(6): 793-8). We have reported that DHMEQ can sensitize drug-resistant tumor cells to drug-induced apoptosis. We examined the differential effects of Salinosporamide A and DHMEQ in their ability to sensitize Daudi to CDDP-induced apoptosis. Tumor cells were treated with different concentrations of DHMEQ (range 1-65 µM) and Salinosporamide A (range 1-50 nM) for 1 h and then treated with CDDP (10 µg/ml) for an additional 20 h and the cells were then examined for apoptosis as described above. The findings in FIG. 3B demonstrate that DHMEQ can sensitize Daudi cells to CDDP-induced apoptosis and sensitization was a function of the concentration used. Interestingly, the extent of sensitization by DHMEQ was similar to that obtained with Salinosporamide A, however, there was a significant difference in the amount of inhibitor used. A 4,000 fold higher concentration used with DHMEQ as compared to Salinosporamide A.

[0133] b) Study with Daudi RR1 Cells

[0134] Similar studies were performed as above in a) with Daudi RR1 cells. The findings in FIG. 4B demonstrate that both inhibitors sensitize cells to CDDP-induced apoptosis. Similar patterns were obtained by both inhibitors; however, there was a 4,000 fold higher concentration used with DHMEQ as compared to Salinosporamide A.

[0135] This finding demonstrates that Salinosporamide A is a superior inhibitor and sensitizing agent as compared to DHMEQ based on the concentration used. However, further studies are needed to demonstrate selectivity with other tumor cell lines.

5) Conclusions:

[0136] The above findings have demonstrated the following:

[0137] 1) Salinosporamide A at very low concentrations (0.1-10 nM) sensitizes both rituximab sensitive and rituximab resistant B-NHL tumor cells to drug-induced apoptosis.

[0138] 2) Comparing the effectiveness of Salinosporamide A and bortezomib in the model system used, revealed that both agents at similar concentrations sensitize B-NHL cells to drug-induced apoptosis.

[0139] 3) Comparing Salinosporamide A and the specific NF-κB inhibitor DHMEQ revealed that both agents sensitized tumor cells to drug-induced apoptosis; however, sensitization by DHMEQ required a 4,000 fold increase in the concentration as compared to Salinosporamide A.

Example II

Salinosporamide a as a Chemotherapeutic Agent for Rituximab-Sensitized Cells

[0140] Our published work with B-NHL cells revealed that rituximab sensitized drug resistant tumor cells to drug induced apoptosis. Sensitization was the result of inhibition of survival pathways such as the Raf-Mek-Erk and NF-κB pathways. These pathways resulted to down regulation of the anti-apoptotic gene product, selectively Bcl_L (Jazirehi and Bonavida, 2005). Since Salinosporamide A was shown to be cytotoxic in sensitive tumor cells, we considered that it might behave like a chemotherapeutic drug and thus we examined whether rituximab can sensitize tumor cells to Salinosporamide A induced apoptosis. We have reported that rituximab treatment of B-NHL cell lines sensitized the drug-resistant cells to drug-induced apoptosis. One of the mechanisms by which rituximab sensitizes the tumor cells to drug-induced apoptosis has been shown to be mediated via inhibition of the NF-κB pathway and downstream the selective inhibition of the anti-apoptotic product BCL_L expression. Inhibitors of this pathway mimicked rituximab in sensitizing the cells to drug-induced apoptosis (Jazirehi, et al, 2005, Cancer Research, 65(1):264-76). We hypothesized that proteasome inhibitors that inhibit NF-κB activity and downstream anti-apoptotic gene products may sensitize tumor cells to drug-induced apoptosis. The new proteasome inhibitor Salinosporamide A (Nereus Pharmaceuticals), which inhibits NF-κB activity, has been shown to sensitize B-NHL cells to drug (CDDP, adriamycin)-induced apoptosis. Salinosporamide A has also been shown to directly kill sensitive tumor cells by apoptosis. Also, Salinosporamide A induces apoptosis in multiple myeloma cells resistant to conventional and bortezomib therapies (Chauhan et al., Cancer Cell 2005 In Press).

[0141] We hypothesized that Salinosporamide A may behave like a chemotherapeutic drug and rituximab may therefore sensitize the tumor cells to Salinosporamide A-induced apoptosis. Ramos cells were treated with rituximab (20 ug/ml) (12 h to 18 h) and the cells were treated with various concentrations of Salinosporamide A (1-10 nM) for an additional 20 h and the cells were examined for apoptosis using the PI method detecting DNA fragmentation by flow cytometry. The combination treatment resulted in significant apoptosis. The synergistic activity was detected with very low concentrations of Salinosporamide A >1 nM. By comparison, several thousand fold higher concentrations of chemotherapeutic drugs (e.g. CDDP, adriamycin) were used for rituximab-mediated chemosensitization of Ramos cells. We also examined the Salinosporamide A resistant Daudi cells following treatment with rituximab for 1 h and Salinosporamide A for an additional 20 h and apoptosis was measured as before. The findings in FIG. 5 show that the combination of rituximab (20 ug/ml) and Salinosporamide A (10 nM) resulted in significant apoptosis and synergy was achieved. These findings demonstrate that the combination of rituximab and Salinosporamide A may be a therapeutic option for the treatment of drug-resistance and resistant tumor cells. FIG. 6 shows that in comparison to rituximab-mediated chemosensitization to CDDP, we have found that rituximab sensitizes to Salinosporamide A-induced apoptosis with a higher level than CDDP. The studies revealed that rituximab can sensitize drug-resistant tumor cells to Salinosporamide A induced apoptosis.

[0142] We further found that rituximab sensitizes cells to Salinosporamide A-induced apoptosis to a higher level than does adriamycin (ADR). As shown in FIG. 8, whereas sole treatment of cells with Salinosporamide A showed no detectable cytotoxic effects, pre-treatment of the tumor cells with rituximab resulted in significant sensitization of the tumor cells to Salinosporamide A-induced apoptosis and synergy was achieved. The sensitization of rituximab to Salinosporamide A-induced apoptosis was greater than that achieved with ADR. FIG. 8 also shows that low concentrations of 1 nM Salinosporamide A-induced significant apoptosis in rituximab pre-treated tumor cells. Higher concentrations of Salinosporamide A (10 nM) appear to be less effective due to cell loss. These results demonstrate that rituximab sensitizes the tumor cells to the proteasome inhibitor Salinosporamide A-mediated apoptosis. In addition, the findings suggest that rituximab (or chemosensitizing agents) used in combination with Salinosporamide A may result in synergistic activity and can reverse drug and/or rituximab resistance of B-NHL.

Example III

Salinosporamide A Induction of Raf-Kinase Inhibitor Protein (RKIP)

[0143] The acquisition of resistance to conventional therapies such as chemotherapy, radiation, and immunotherapy remains a major obstacle in the successful treatment of cancer. Among the mechanisms of resistance is the acquisition of resistance to apoptotic stimuli by tumor cells. Hence, tumor cells develop mechanisms to resist apoptosis and exhibit constitutive hyperactivation of survival and anti-apoptotic signaling pathways. Tumor suppressors exist in normal cells that negatively regulate cell survival and enhance response to apoptotic stimuli. The dysregulation of such controls that regulate cell survival and proliferation leads to neoplastic

transformation. Thus, most tumor cells have dysregulated expression or function of functional tumor suppressors through deletion or mutation or low expression. Therefore, agents that can upregulate the expression of functional tumor suppressors would be useful to counteract the survival and anti-apoptotic pathways in tumor cells. Such agents would be expected to inhibit tumor cell proliferation or survival and/or sensitize cells to the cytotoxic effect of conventional cytotoxic therapies.

[0144] As shown in FIG. 9, treatment of tumor cells with Salinosporamide A results in the induction of the tumor suppressor, Raf-kinase inhibitor protein (RKIP). RKIP is a member of the phosphatidylethanolamine-binding protein (PEBP) family. Ramos cells were treated with 10 nM Salinosporamide A for various periods of time, and total cell lysates were examined for the expression of RKIP by western blot analysis. The expression of β -actin was used as a control. The data in FIG. 9 shows that there is an increase in the levels of RKIP protein after a 15 minute exposure of Ramos cells to 10 nM Salinosporamide A.

[0145] It has been shown that RKIP inhibits the Raf/MEK/ERK 1/2 and the NF- κ B survival signaling pathways, and consequently, the expression of several anti-apoptotic gene products that are regulated by these pathways. Furthermore, expression of RKIP has been shown to reverse the resistance of drug-resistant cancer cells to drug-induced apoptosis. In addition, RKIP expression has been found to be depressed in primary tumors as compared to normal tissues and has been found to be lost following malignancy and metastasis in tumors. Thus, the ability of Salinosporamide A to induce the expression of RKIP in tumor cells provides a novel therapeutic target for avoiding or reversing therapy resistance of cancer cells and may be especially useful in treating metastases.

Example IV

Salinosporamide A Inhibits the Expression of YY1

[0146] As shown in FIG. 10, treatment of tumor cells with Salinosporamide A results in the inhibition of expression of the transcriptional regulator protein YY1. YY1 is a transcription repressor that is overexpressed in cancer cells and has been shown to play a role in maintaining the resistance of tumor cells to various therapeutics. Ramos cells were treated with various concentrations of Salinosporamide A for 24 hours, and total cell lysates were examined for the expression of YY1 by western blot analysis and by RT-PCR. The expression of β -actin was used as a control. The data in FIG. 10 shows that there is a decrease in the levels of YY1 protein after a 24 hour exposure of Ramos cells to various concentrations of Salinosporamide A.

Example V

Salinosporamide A Analogs

[0147] Analogs of Salinosporamide A, as shown in Formula I and in US 20050049294 are tested for activity as sensitizing agents and as chemotherapeutic agents as described above in Examples I, II, and III.

Example VI

Salinosporamide A Activates Caspase 9 and Inhibits Bcl-XL Expression

[0148] FIGS. 13 and 14 demonstrate that Salinosporamide A treatment of tumor cells results in the activation of caspase

9, and also inhibits expression of the anti-apoptotic gene BCLxL. Activation of caspase 9 indicates that Salinosporamide A activates the mitochondria and type II apoptosis and facilitates its direct or indirect activation of the effector caspases 3 and 7 for apoptosis. The inhibition of BCLxL by Salinosporamide A demonstrates that it inhibits a key anti-apoptotic factor that regulates resistance in many tumors. Also, it suggests that Salinosporamide A mediated inhibition of BCLxL may be responsible, in part, for its sensitizing effect on resistant tumor cells.

Example VII

Salinosporamide A Induces PTEN and Inhibits PTN

[0149] The findings in FIG. 15 demonstrate that Salinosporamide A induces expression of the phosphatase inhibitor PTEN, or the AKT cell survival pathway. This induction inhibits the anti-apoptotic AKT pathway and contributes to Salinosporamide A induced sensitization to apoptosis. Further, most resistant tumor cells express low levels of PTEN, and PTEN is a target for therapeutic intervention, as well as a biomarker

[0150] The findings in FIG. 16 demonstrate that Salinosporamide A inhibits the expression of the growth factor pleiotrophin (PTN). PTN has been reported to be elevated in tumor cells and in the circulation of cancer patients and is of prognostic significance. In addition, inhibition of PTN contributes to the sensitizing effect of Salinosporamide A. PTN is also a target for therapeutic intervention.

Example VII

Salinosporamide A Upregulates Death Receptor DR5 and Sensitizes Tumor Cells to Trail-Induced Apoptosis

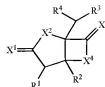
[0151] FIGS. 17 A and B, 18 and 19 demonstrate that Salinosporamide A upregulates the expression of the TRAIL death receptor DR5 and sensitizes TRAIL resistant tumor cells to TRAIL mediated apoptosis. These findings demonstrate also that Salinosporamide A is a therapeutic agent that can be used in combination with TRAIL or agonist anti-DR5/DR5 mAbs in the treatment of drug/TRAIL resistant cells.

REFERENCES

- [0152]** Feling, et al., *Angew. Chem. Int. Ed.*, 2003, 42(3): 355-357
- [0153]** Horiguchi, et al., *Expert Rev. Anticancer Ther.*, 2003, 3(6): 793-8.
- [0154]** Jazirehi and Bonavida, *Oncogene*. 2005, 24(13): 2121-43.
- [0155]** Macherla, et al., *Journal of Medicinal Chemistry*, 2005, 48:3684
- [0156]** Suzuki, et al., AACR Annual Meeting, Abstract number 5429, Apr. 1-5, 2006.
- [0157]** It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

1. A method of treating, preventing or inhibiting a cancer, the method comprising the step of administering to a subject

a therapeutically effective amount of a cancer therapy reagent and a sensitizingly effective amount of a compound of Formula I:



wherein

each of R¹, R² and R³ are independently selected from the group consisting of H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, —C(O)H, acyl, oxycyl, carbamate, sulfonfyl, sulfonamide, and sulfuryl;

R⁴ is a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁶ groups;

each R⁵ is independently selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, and substituted cycloalkyl;

each of X¹, X², X³ and X⁴ is independently selected from the group consisting of O, NR⁶ and S; and

R⁶ is H or C₁-C₆ alkyl.

2. The method of claim 1, wherein

each of R¹ and R² are independently selected from the group consisting of H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, and substituted cycloalkyl;

R³ is selected from the group consisting of alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, oxycyl, carbamate, sulfonfyl, sulfonamide, and sulfuryl;

R⁴ is a 5-8 membered cycloalkenyl optionally substituted with 1-8 R⁵ groups;

each of X¹, X³ and X⁴ is O; and

X² is NH.

3. The method of claim 1, wherein

each of R¹ and R² are independently selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, and substituted alkynyl;

R³ is selected from the group consisting of alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, and amino;

R⁴ is cyclohexenyl optionally substituted with 1-8 R⁵ groups;

each of X¹, X³ and X⁴ is O; and

X² is NH.

4. The method of claim 1, wherein

R¹ is an alkyl or substituted alkyl;

R² is alkyl;

R³ is hydroxy;

R⁴ is cyclohexenyl; and

each of X¹, X³ and X⁴ is O; and

X² is NH.

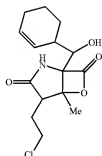
5. The method of claim 4, wherein the substituted alkyl of R^1 is a halogenated alkyl.

6. The method of claim 5, wherein the halogenated alkyl is selected from the group consisting of a fluorinated alkyl chlorinated alkyl, and brominated alkyl.

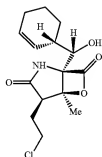
7. (canceled)

8. (canceled)

9. The method of claim 7, wherein the compound has the following structure:



10. The method of claim 7, wherein the compound has the following structure:



11. The method of claim 1, wherein the sensitizingly effective amount of the compound of Formula I is sufficient to inhibit expression of an anti-apoptotic gene product, thereby inducing apoptosis.

12. (canceled)

13. (canceled)

14. (canceled)

15. (canceled)

16. (canceled)

17. (canceled)

18. (canceled)

19. (canceled)

20. The method of claim 1, wherein the cancer therapy reagent is a chemotherapeutic reagent, an immunotherapeutic reagent, a radiotherapeutic reagent, or a hormonal therapeutic reagent.

21. The method of claim 1, wherein the cancer is selected from the group consisting of: non-Hodgkin's lymphoma, B-acute lymphoblastic lymphoma, prostate cancer, ovarian

cancer, renal cancer, lung cancer, breast cancer, colon cancer, leukemia, multiple myeloma and hepatocarcinoma.

22. The method of claim 21, wherein the cancer is lymphoma.

23. The method of claim 21, wherein the cancer is non-Hodgkin's lymphoma.

24. The method of claim 1, wherein the cancer therapy reagent induces apoptosis.

25. The method of claim 24, wherein the cancer therapy reagent is a chemotherapeutic reagent, an immunotherapeutic reagent, a radiotherapeutic reagent, or a hormonal therapeutic reagent.

26. The method of claim 24, wherein the cancer therapy reagent is rituximab immunotherapy.

27. The method of claim 26, wherein the cancer is B-NHL.

28. (canceled)

29. The method of claim 1, wherein the cancer is therapy-resistant.

30. The method of claim 29, wherein the therapy is selected from the group consisting of immunotherapy, chemotherapy, radiotherapy, and hormonal therapy.

31. The method of claim 1, wherein the cancer is therapy-sensitive.

32. The method of claim 1, wherein the therapeutically effective amount of a cancer therapy reagent and the sensitizingly effective amount of a compound of Formula I are administered concurrently.

33. The method of claim 32, wherein the cancer therapy reagent comprises bortezomib administration.

34. The method of claim 32, wherein the cancer therapy reagent is a chemotherapeutic reagent, an immunotherapeutic reagent, a radiotherapeutic reagent, or a hormonal therapeutic reagent.

35. The method of claim 1, wherein the therapeutically effective amount of a cancer therapy reagent and the sensitizingly effective amount of a compound of Formula I are administered sequentially.

36. The method of claim 1, wherein the subject is a human.

37. (canceled)

38. (canceled)

39. (canceled)

40. (canceled)

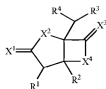
41. (canceled)

42. (canceled)

43. A composition comprising a therapeutically effective amount of rituximab and a sensitizingly effective amount of a compound of Formula I in a physiologically acceptable excipient.

44. A kit comprising a therapeutically effective amount of rituximab and a sensitizingly effective amount of a compound of Formula I.

45. A method of treating, preventing or inhibiting a cancer with proteasome inhibitor therapy, the method comprising the step of administering to a subject a sensitizingly effective amount of an antibody or chemosensitizing reagent and a therapeutically effective amount of a compound of Formula I:



wherein

each of R^1 , R^2 and R^3 are independently selected from the group consisting of H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, $-C(O)H$, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, and sulfuryl;

R^4 is a 5-8 membered cycloalkyl optionally substituted with 1-8 R^5 groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R^5 groups;

each R^5 is independently selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, and substituted cycloalkyl;

each of X^1 , X^2 , X^3 and X^4 is independently selected from the group consisting of O, NR^6 and S; and

R^6 is H or C_1-C_6 alkyl.

46. (canceled)

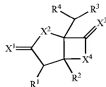
47. The method of claim 45, wherein the antibody is rituximab.

48. The method of claim 45, wherein the cancer is lymphoma.

49. A composition comprising a sensitizingly effective amount of rituximab and a therapeutically effective amount of a compound of Formula I in a physiologically acceptable excipient.

50. A kit comprising a sensitizingly effective amount of rituximab and a therapeutically effective amount of a compound of Formula I.

51. A method of treating, preventing or inhibiting a cancer, the method comprising the step of administering to a subject a therapeutically effective amount of a compound of Formula I:



wherein

each of R^1 , R^2 and R^3 are independently selected from the group consisting of H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, het-

erocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, $-C(O)H$, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, and sulfuryl;

R^4 is a 5-8 membered cycloalkyl optionally substituted with 1-8 R^5 groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R^5 groups;

each R^5 is independently selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, and substituted cycloalkyl;

each of X^1 , X^2 , X^3 and X^4 is independently selected from the group consisting of O, NR^6 and S; and

R^6 is H or C_1-C_6 alkyl, and

wherein said therapeutically effective amount is sufficient to induce the expression of RKIP or PTEN, thereby inducing apoptosis.

52. (canceled)

53. (canceled)

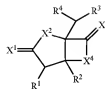
54. (canceled)

55. (canceled)

56. (canceled)

57. (canceled)

58. A method of treating a therapy resistant cancer, the method comprising the step of administering to a subject a therapeutically effective amount of a compound of Formula I:



wherein

each of R^1 , R^2 and R^3 are independently selected from the group consisting of H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, alkoxy, substituted alkoxy, thioalkyl, substituted thioalkyl, hydroxy, halogen, amino, amido, carboxyl, $-C(O)H$, acyl, oxyacyl, carbamate, sulfonyl, sulfonamide, and sulfuryl;

R^4 is a 5-8 membered cycloalkyl optionally substituted with 1-8 R^5 groups or a 5-8 membered cycloalkenyl optionally substituted with 1-8 R^5 groups;

each R^5 is independently selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, and substituted cycloalkyl;

each of X^1 , X^2 , X^3 and X^4 is independently selected from the group consisting of O, NR^6 and S; and

R^6 is H or C_1-C_6 alkyl, and

wherein said therapeutically effective amount is sufficient to induce the expression of RKIP or PTEN, thereby inducing apoptosis.

* * * * *



US 20090156469A1

(19) **United States**(12) **Patent Application Publication**
Ghobrial et al.(10) **Pub. No.: US 2009/0156469 A1**(43) **Pub. Date: Jun. 18, 2009**(54) **METHODS OF USING [3.2.0]
HETEROCYCLIC COMPOUNDS AND
ANALOGS THEREOF IN TREATING
WALDENSTROM'S MACROGLOBULINEMIA**(22) Filed: **Dec. 5, 2008****Related U.S. Application Data**

(60) Provisional application No. 61/012,396, filed on Dec. 7, 2007.

Publication Classification(51) **Int. Cl.***A61K 38/14* (2006.01)*A61K 31/40* (2006.01)*A61K 31/69* (2006.01)*A61K 31/439* (2006.01)*A61P 35/00* (2006.01)*C12N 5/00* (2006.01)*A61K 38/07* (2006.01)*A61K 31/704* (2006.01)(52) **U.S. Cl. 514/8; 514/421; 514/34; 514/64;
514/18; 514/290; 435/375**(57) **ABSTRACT**

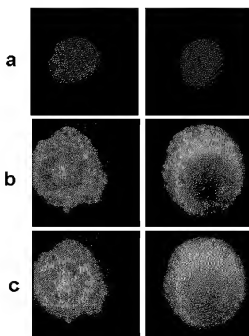
Disclosed are methods of treating Waldenstrom's Macroglobulinemia comprising administering to the animal, a therapeutically effective amount of a heterocyclic compound of Formula I.

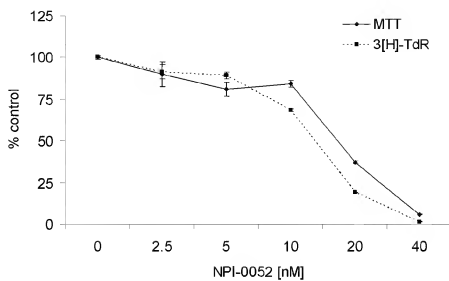
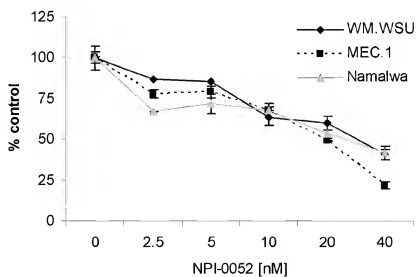
(75) Inventors: **Irene Ghobrial**, Chestnut Hill, MA (US); **Aldo Roccaro**, Boston, MA (US); **Dharminder Chauhan**, Natick, MA (US); **Kenneth Anderson**, Wellesley, MA (US); **Michael A. Palladino**, Olivenhain, CA (US)

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IRVINE, CA 92614 (US)**(73) Assignees: **NEREUS
PHARMACEUTICALS, INC.**, San Diego, CA (US); **Dana-Farber Cancer Institute**, Boston, MA (US)(21) Appl. No.: **12/329,504**

TNF- α	+	+
Bortezomib	-	+
NPI-0052	-	+



**FIG. 1A****FIG. 1B**

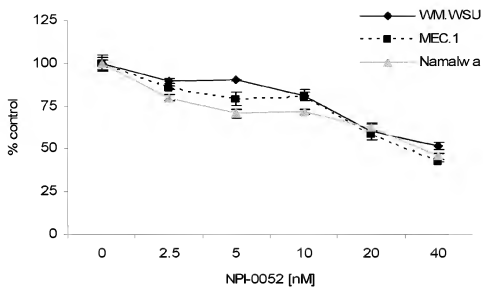


FIG. 1C

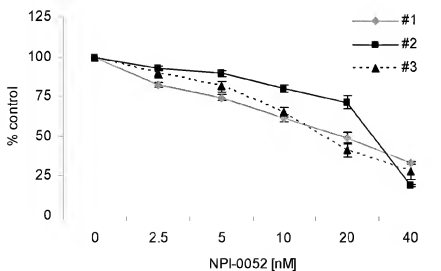


FIG. 1D

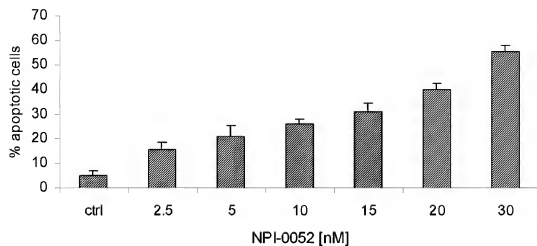


FIG. 1E

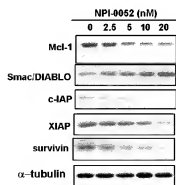
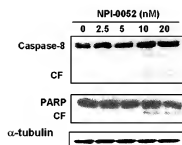


FIG. 1F

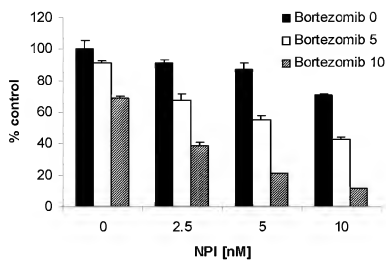


FIG. 2A

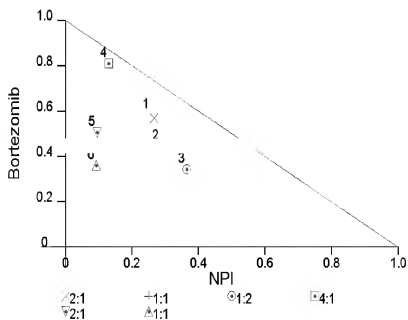
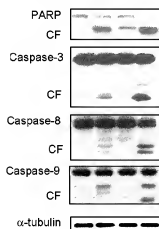


FIG. 2B

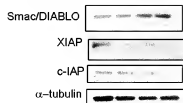
B (nM)	N (nM)	F.A.	C.I.
5	2.5	0.25	0.835
5	5	0.39	0.727
5	10	0.53	0.709
10	2.5	0.43	0.941
10	5	0.69	0.601
10	10	0.83	0.454

FIG. 2C

Bortezomib - + - +
NPI-0052 - - + +



Bortezomib - + - +
NPI-0052 - - + +



Bortezomib - + - +
NPI-0052 - - + +

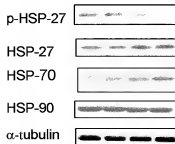
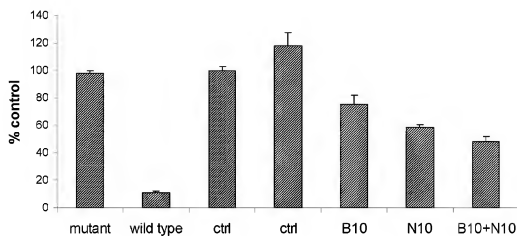
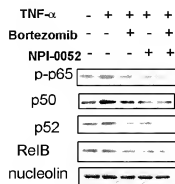


FIG. 2D

**FIG. 3A****FIG. 3B**

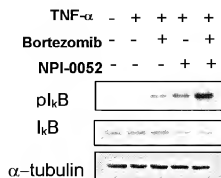


FIG. 3C

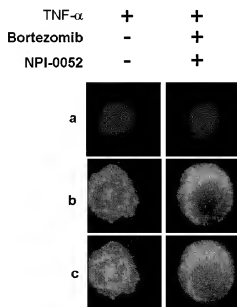


FIG. 3D

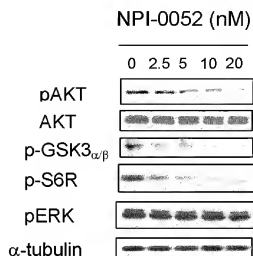


FIG. 4A

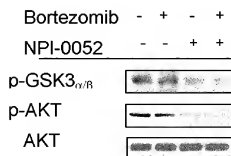
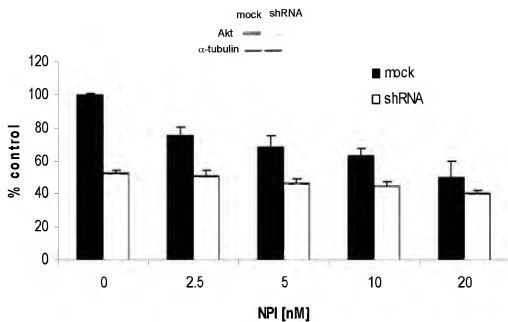
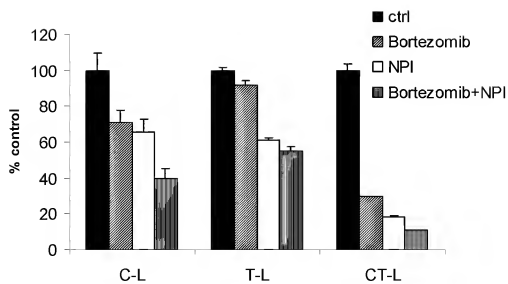
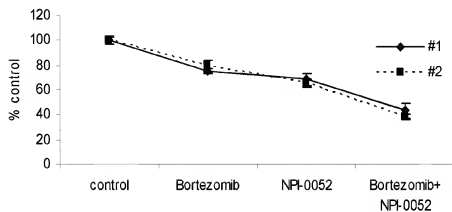
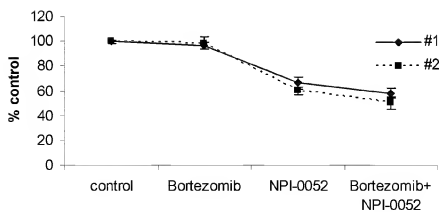


FIG. 4B

**FIG. 4C****FIG. 4D**

**FIG. 4Ei****FIG. 4Eii**

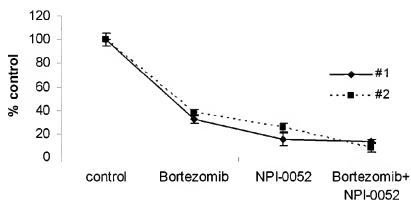


FIG. 4Eiii

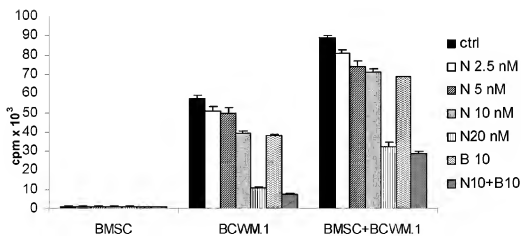
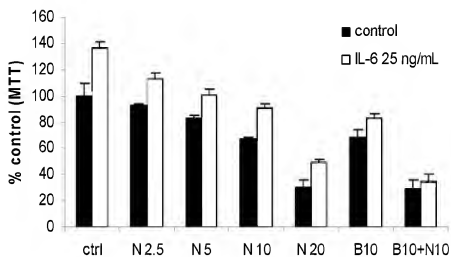
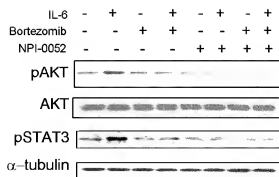


FIG. 5A

**FIG. 5B****FIG. 5C**

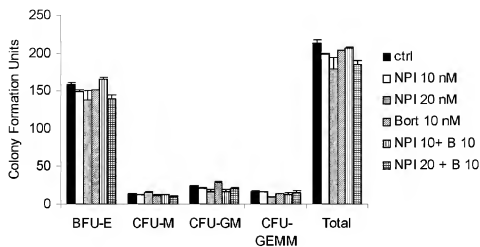


FIG. 5D

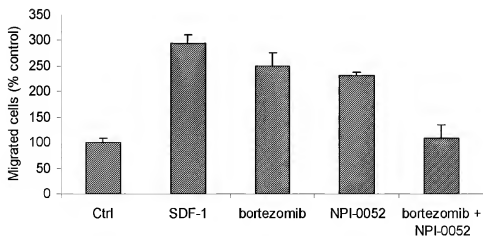


FIG. 6A

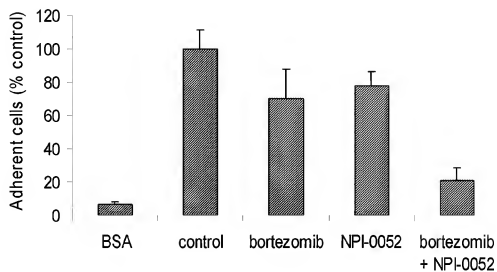


FIG. 6B

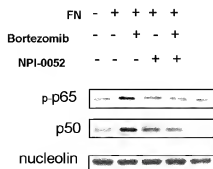
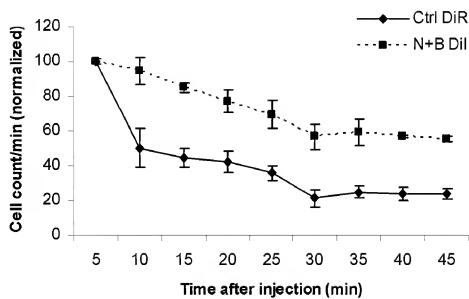


FIG. 6C

**FIG. 6D**

METHODS OF USING [3,2,0] HETEROCYCLIC COMPOUNDS AND ANALOGS THEREOF IN TREATING WALDENSTROM'S MACROGLOBULINEMIA

RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 61/010126, filed Dec. 7, 2007, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The present invention relates to certain compounds and to methods for the preparation and the use of certain compounds in the fields of chemistry and medicine.

[0004] 2. Description of the Related Art

[0005] Cancer is a leading cause of death in the United States. Despite significant efforts to find new approaches for treating cancer, the primary treatment options remain surgery, chemotherapy and radiation therapy, either alone or in combination. Surgery and radiation therapy, however, are generally useful only for fairly defined types of cancer, and are of limited use for treating patients with disseminated disease. Chemotherapy is the method that is generally useful in treating patients with metastatic cancer or diffuse cancers such as leukemias. Although chemotherapy can provide a therapeutic benefit, it often fails to result in cure of the disease due to the patient's cancer cells becoming resistant to the chemotherapeutic agent. Due, in part, to the likelihood of cancer cells becoming resistant to a chemotherapeutic agent, such agents are commonly used in combination to treat patients.

[0006] Waldenström's Macroglobulinemia (WM) is a biologically unique low-grade B-cell lymphoma. WM is characterized by the presence of lymphoplasmacytic cells in the bone marrow and the secretion of IgM monoclonal protein in the serum, indicating that WM cells find a high protein turnover. WM is becoming a model low-grade lymphoma to test and validate therapeutic compounds that are specifically active in this biologically unique malignancy.

[0007] Therefore, a need exists for additional chemotherapeutic agents to treat cancer and more specifically WM. A continuing effort is being made by individual investigators, academia and companies to identify new, potentially useful chemotherapeutic agents.

[0008] Marine-derived natural products are a rich source of potential new anti-cancer agents and anti-microbial agents. The oceans are massively complex and house a diverse assemblage of microbes that occur in environments of extreme variations in pressure, salinity, and temperature. Marine microorganisms have therefore developed unique metabolic and physiological capabilities that not only ensure survival in extreme and varied habitats, but also offer the potential to produce metabolites that would not be observed from terrestrial microorganisms (Okami, Y. 1993 *J Mar Biotechnol* 1:59). Representative structural classes of such metabolites include terpenes, peptides, polyketides, and compounds with mixed biosynthetic origins. Many of these molecules have demonstrable anti-tumor, anti-bacterial, anti-fungal, anti-inflammatory or immunosuppressive activities (Bull, A. T. et al. 2000 *Microbiol Mol Biol Rev* 64:573; Cragg, G. M. & D. J. Newman 2002 *Trends Pharmacol Sci* 23:404; Kerr, R. G. & S. S. Kerr 1999 *Exp Opin Ther Patents* 9:1207; Moore, B. S. 1999 *Nat Prod Rep* 16:653; Faulkner, D. J. 2001

Nat Prod Rep 18:1; Mayer, A. M. & V. K. Lehmann 2001 *Anticancer Res* 21:2489), validating the utility of this source for identifying invaluable therapeutic agents. Further, the isolation of novel anti-cancer and anti-microbial agents that represent alternative mechanistic classes to those currently on the market will help to address resistance concerns, including any mechanism-based resistance that may have been engineered into pathogens for bioterrorism purposes.

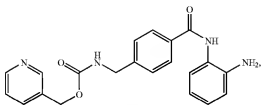
SUMMARY OF THE INVENTION

[0009] The embodiments disclosed herein generally relate to chemical compounds, including heterocyclic compounds and analogs thereof.

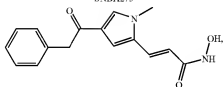
[0010] In some embodiments, the compounds are used to treat WM. Certain embodiments relate to methods of treating WM in animals. The method can include, for example, administering an effective amount of a compound to a patient in need thereof. Other embodiments relate to the use of compounds in the manufacture of a pharmaceutical or medication for the treatment of WM.

[0011] The compounds can be administered or used in combination with treatments such as chemotherapy, radiation, and biologic therapies. In some embodiments the compounds can be administered or used with a chemotherapeutic agent. Examples of such chemotherapeutics include alkylating agents, antibiotics, antimetabolites, enzymes, hormones, platinum compounds, immunotherapeutics (antibodies, T-cells, epitopes), BRMs, and the like. Examples include, Vincristine, Vinblastine, Vindesine, Paclitaxel (Taxol), Docetaxel, topoisomerase inhibitors epipodophyllotoxins (Etoposide (VP-16), Teniposide (VM-26)), Camptothecin, nitrogen mustards (cyclophosphamide, Cytosan), Nitrosoureas, Carmustine, lomustine, dacarbazine, hydroxymethylmelamine, thiopeta and mitocycin C, Dactinomycin (Actinomycin D), anthracycline antibiotics (Daunorubicin, Daunomycin, Cerubidine), Doxorubicin (Adriamycin), Idarubicin (Idamycin), Anthracenediones (Mitoxantrone), Bleomycin (Blenoxane), Plicamycin (Mithramycin, Antifolates (Methotrexate (Folex, Mexate)), purine antimetabolites (6-mercaptopurine (6-MP, Purinethol) and 6-thioguanine (6-TG)). The two major anticancer drugs in this category are 6-mercaptopurine and 6-thioguanine. Chlorodeoxyadenosine and Pentostatin, Pentostatin (2'-deoxycofomycin), pyrimidine antagonists, Avastin, Leucovorin, Oxaliplatin, fluoropyrimidines (5-fluorouracil (Adrucil), 5-fluorodeoxyuridine (FdUrd) (Flouxuridine)), Cytosine Arabinoside (Cytosar, ara-C), Fludarabine, L-ASPARAGINASE, Hydroxyurea, glucocorticoids, antiestrogens, tamoxifen, nonsteroidal antiandrogens, flutamide, aromatase inhibitors Anastrozole (Arimidex), Cisplatin, 6-Mercaptopurine and Thioguanine, Methotrexate, Cytosar, Cytarabine, L-Asparaginase, Steroids: Prednisone and Dexamethasone. Also, proteasome inhibitors such as bortezomib and carfilzomib (PR-171) can be used in combination with the instant compounds, for example. Examples of biologics can include agents such as TRAIL, antibodies to TRAIL and agonistic antibodies TRAIL, death receptors DR4 and DR5, integrins such as alpha-V-beta-3 ($\alpha V\beta 3$) and/or other cytokine/growth factors that are involved in angiogenesis, VEGF, EGF, FGF and PDGF and antibodies to these cytokines/growth factors such as Eribulin. In some aspects, the compounds can be conjugated to or delivered with an antibody.

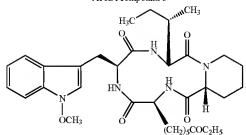
[0012] In other embodiments, the compounds are administered in combination with a histone deacetylase inhibitor (HDACi). In various embodiments, the HDACi is selected from the group consisting of:



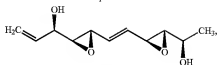
SNDX275



APHA compound 8



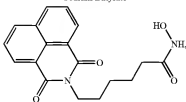
Apicidin



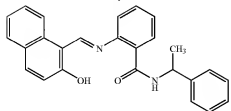
(-)-Depudecin



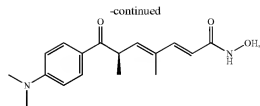
Sodium Butyrate



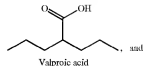
Scriptaid



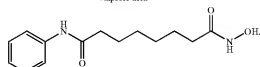
Sirtinol



Trichostatin A

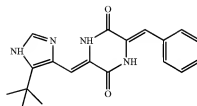


Valproic acid



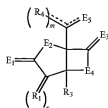
Vorinostat (suberoylanilide hydroxamic acid (SAHA))

[0013] In other embodiments, the compounds can be used in combination with vascular disrupting agents (VDA). Examples of such VDAs include combrostatin CA4P and NPI-2358. NPI-2358 is represented by the following formula:



NPI-2358

[0014] Some embodiments relate to uses of a structure of Formula I, or a pharmaceutically acceptable salt or pro-drug ester thereof:



Formula I

[0015] wherein R_1 , R_2 , and R_3 separately may include a hydrogen, a halogen, a mono-substituted, a poly-substituted or an unsubstituted variant of the following residues: saturated $C_{1-C_{24}}$ alkyl, unsaturated $C_{2-C_{24}}$ alkenyl or $C_{2-C_{24}}$ alkynyl, acyl, acyloxy, alkoxy, alkoxy carbonyloxy, aryloxy, carbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy carbonyl, alkoxy carbonyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxy sulfonyl, car-

boxy, cyano, thio, sulfoxide, sulfone, sulfonate esters, thiocyanate, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0016] wherein *m* is equal to 1 or 2;

[0017] wherein *n* is equal to 1 or 2;

[0018] wherein each of E₁, E₂, E₃ and E₄ is a substituted or unsubstituted heteroatom; and

[0019] wherein E₅ may include OH, O, OR₁₀, S, SR₁₁, SO₂, R₁₁, NH, NH₂, NOH, NHOH, NR₁₂, and NHOR₁₃.

[0020] Other embodiments relate to methods of treating WM in an animal. The methods can include, for example, administering to the animal, a therapeutically effective amount of a compound of a formula selected from Formula I, and pharmaceutically acceptable salts and pro-drug esters thereof.

[0021] Further embodiments relate to pharmaceutical compositions which include a compound of a formula selected from Formula I.

[0022] Still further embodiments relate to methods of inhibiting the growth of a cancer cell. The methods can include, for example, contacting a cancer cell with a compound of a formula selected from Formula I and pharmaceutically acceptable salts and pro-drug esters thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] The accompanying drawings, which are incorporated in and form part of the specification, merely illustrate certain preferred embodiments of the present invention. Together with the remainder of the specification, they are meant to serve to explain preferred modes of making certain compounds of the invention to those of skill in the art. In the drawings:

[0024] FIG. 1A is a graph showing thymidine uptake assay and cytotoxicity assessed by MTT. BCWM.1 cells were cultured with salinosporamide A (2.5-40 nM) for 48 hours.

[0025] FIG. 1B is a graph showing thymidine uptake assay. Several IgM-secreting cell lines, WM-WSU (◆), MEC-1 (■), Namalwa were cultured with salinosporamide A (2.5-40 nM) for 48 hours.

[0026] FIG. 1C is a graph showing several IgM secreting cell lines, WM-WSU, MEC-1, Namalwa were cultured with salinosporamide A for 48 hours. Cytotoxicity was assessed by MTT assay.

[0027] FIG. 1D is a graph showing freshly isolated bone marrow CD19⁺ tumor cells from 3 patients with WM were cultured with salinosporamide A (2.5-40 nM [E]; 2.5-80 nM [F]). Cytotoxicity was assessed by MTT assay.

[0028] FIG. 1E is a graph showing BCWM.1 cultured with salinosporamide A for 48 hours at doses that range from 2 to 30 nM and the percentage of cells undergoing apoptosis was studied by Apo2.7 staining.

[0029] FIG. 1F is a western blot showing BCWM.1 cells cultured with salinosporamide A (2.5-20 nM) for 12 hours. Whole cell lysates were subjected to Western blotting using anti-caspase 8, -PARP, -Mcl-1, -Smac/DIABLO, -cIAP, -XIAP, -survivin, and -α-tubulin antibodies.

[0030] FIG. 2A is a graph showing BCWM.1 cells cultured with salinosporamide A (2.5, 5 and 10 nM) for 48 hours, in the presence or absence of bortezomib (5 and 10 nM). Cytotoxicity was assessed by MTT assay.

[0031] FIG. 2B is a graph showing a representative isobologram of salinosporamide A associated to bortezomib with the CalcuSyn software demonstrating synergy for the combination.

[0032] FIG. 2C is a chart showing indexes (C.I.) and fractions affected (FA) of the combinations of salinosporamide A and bortezomib. All experiments were repeated in triplicate.

[0033] FIG. 2D is a picture showing BCWM.1 cultured with salinosporamide A (10 nM) in presence or absence of bortezomib (10 nM) for 12 hours. Whole cell lysates were subjected to Western blotting using anti-caspase-8, -9, -3, -PARP, -Smac/DIABLO, -cIAP, -survivin, -p-HSP27, -HSP27, -HSP70, -HSP90 and α-tubulin antibodies.

[0034] FIG. 3A is a graph showing BCWM.1 cells cultured with either salinosporamide A (10 nM), bortezomib (10 nM), or the combination for 4 hours, then human TNF-α (10 ng/mL) was added for the last 20 minutes. NF-κBp65 transcription factor-binding to its consensus sequence on the plate-bound oligonucleotide was studied from nuclear extract. Wild type and mutant are wild type and mutated consensus competitor oligonucleotides, respectively. All results represent means (±sd) of triplicate experiments.

[0035] FIG. 3B is a picture showing BCWM.1 cells cultured with either salinosporamide A (10 nM), bortezomib (10 nM), or the combination for 4 hours, and TNF-α (10 ng/mL) was added for the last 20 minutes. Cytoplasmic and nuclear extracts were subjected to western blotting using anti-p-NF-κBp65, -NF-κBp50, -NF-κBp52 IκBα, -RelB, -p-IκB, -IκB, -nucleolin and -α-tubulin antibodies.

[0036] FIG. 3C is a picture showing BCWM.1 cells cultured with either salinosporamide A (10 nM), bortezomib (10 nM), or the combination for 4 hours, and TNF-α (10 ng/mL) was added for the last 20 minutes. Cytoplasmic and nuclear extracts were subjected to western blotting using anti-p-NF-κBp65, -NF-κBp50, -NF-κBp52 IκBα, -RelB, -p-IκB, -IκB, -nucleolin and -α-tubulin antibodies.

[0037] FIG. 3D is a picture showing BCWM.1 cells cultured with salinosporamide A (10 nM) and bortezomib (10 nM) for 4 hours, or control medium, and TNF-α (10 ng/mL) was added for the last 20 minutes. Immunocytochemical analysis was assessed as described in Materials and Methods.

[0038] FIG. 4A is a picture showing BCWM.1 cultured with salinosporamide A (2.5-20 nM) for 6 hours. Whole cell lysate were subjected to Western blotting using anti-p-Akt, -Akt, -p-GSK3α/β, -p-S6R, -p-ERK, and -α-tubulin antibodies.

[0039] FIG. 4B is a picture showing In vitro Akt kinase assay. BCWM.1 cells cultured with control media or salinosporamide A (2.5-20 nM) for 6 hours. Whole cell lysates were immunoprecipitated with anti-Akt antibody. Then the immunoprecipitate was washed and subjected to in vitro kinase assay according to manufacturer's protocol. Western blotting used anti-p-GSK3α/β and anti-Akt antibodies.

[0040] FIG. 4C is a graph showing BCWM.1 cells transduced with Akt shRNA for 48 hours. Mock: control plasmid. BCWM.1 transfected cells or BCWM.1 control cells were treated with salinosporamide A (2.5-20 nM) for 48 hours. Cytotoxicity was assessed by MTT assay. Whole cell lysates were subjected to western blotting using anti-p-Akt, -Akt, and α-tubulin antibodies.

[0041] FIG. 4D is a graph showing BCWM.1 cells or primary CD19⁺ tumor cells from 2 patients with WM (FIG. 4E i, ii, iii) were incubated for 4 hours in the presence of diluent or 10 nM salinosporamide A, bortezomib 10 nM, or bortezomib+salinosporamide A. The chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L) activity of the 20S proteasome of BCWM.1 was determined by measurement of

fluorescence generated from the cleavage of the fluorogenic substrates suc-1-LVY-ame, boc-LRR-ame, and z-LLE-ame, respectively.

[0042] FIG. 5A is a graph showing BCWM.1 cells cultured with control media, and with salinosporamide A (2.5-20 nM), with and without bortezomib (10 nM) for 48 hours, in the presence or absence of BMSCs. Cell proliferation was assessed using [³H]-thymidine uptake assay. All data represent mean (±sd) of triplicate experiment.

[0043] FIG. 5B is a graph showing BCWM.1 cultured with control media or salinosporamide A (2.5-20 nM), with and without bortezomib (10 nM) for 48 hours, in presence or absence of IL-6 (25 ng/mL) (10 μM). Proliferation was assessed by thymidine uptake assay.

[0044] FIG. 5C is a western blot showing BCWM.1 cells cultured with control media or salinosporamide A (10 nM) with and without bortezomib (10 nM) for 8 hours. Cells were then stimulated with IL-6 (25 ng/mL) for 10 minutes. Whole cell lysates were subjected to western blotting using anti-p-AKT, anti-AKT, anti-p-STAT3 and anti-α-tubulin.

[0045] FIG. 5D is a graph showing colony-forming cell assay. Negative fraction after CD19⁺ selection of bone marrow mononuclear cells was cultured using methylcellulose semisolid technique in absence or presence of salinosporamide A (10 nM, 20 nM) either alone or in combination with bortezomib 10 nM, and BFU-E, CFU-GM, CFU-M and CFU-GEMM were counted at day 14th. All experiments have been done in triplicate.

[0046] FIG. 6A is a graph showing Transwell migration assay showing inhibition of migration of BCWM.1 in the presence of salinosporamide A (2.5-20 nM), bortezomib (10 nM), or salinosporamide A (10 nM) in combination with bortezomib (10 nM). SDF-1 30 nM was placed in the lower chambers and induced migration as compared to control with no SDF-1 (Ctrl, control). SDF-1 was placed in the lower chambers of the salinosporamide A/bortezomib-treated wells.

[0047] FIG. 6B is a graph showing an adhesion assay with BCWM.1 cells in the presence or absence of salinosporamide A (10 nM), either alone or in combination with bortezomib (10 nM). BCWM.1 cells demonstrated increased adhesion in fibronectin-coated wells (control) as compared to BSA-coated wells (BSA, bovine serum albumin). All data represent mean (±sd) of triplicate experiments.

[0048] FIG. 6C is a picture showing BCWM.1 cells cultured with control media or salinosporamide A (10 nM) with and without bortezomib (10 nM) for 4 hours, in presence or absence of fibronectin (FN). Nuclear extracts were subjected to western blotting using anti-p-p65, p50, and -nucleolin antibodies.

[0049] FIG. 6D is a graph showing in vivo flow cytometry. DiI-labeled treated cells and DiR-labeled untreated cells were injected in the tail vein of BALB/c mice. Cells were counted every 5 min for 45 minutes as described in Material and Methods.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0050] Numerous references are cited herein. The references cited herein, including the U.S. patents cited herein, are each to be considered incorporated by reference in their entirety into this specification.

[0051] Embodiments of the invention include, but are not limited to, providing a method for the preparation of com-

pounds, including compounds, for example, those described herein and analogs thereof, and to providing a method for producing pharmaceutically acceptable anti-cancer compositions. The methods can include the compositions in relatively high yield, wherein the compounds and/or their derivatives are among the active ingredients in these compositions. Other embodiments relate to providing novel compounds not obtainable by currently available methods. Furthermore, embodiments relate to methods of treating cancers, particularly those affecting humans. In some embodiments, one or more formulae, one or more compounds, or groups of compounds can be specifically excluded from use in any one or more of the methods of treating the conditions described herein. As one illustrative example, compounds of Formula 1-16 can be excluded in some embodiments from the methods of treating cancer generally, for example, or a specific type of cancer. The methods may include, for example, the step of administering an effective amount of a member of a class of new compounds. Preferred embodiments relate to the compounds and methods of making and using such compounds disclosed herein, but not necessarily in all embodiments of the present invention, these objectives are met.

[0052] For the compounds described herein, each stereogenic carbon can be of R or S configuration. Although the specific compounds exemplified in this application can be depicted in a particular configuration, compounds having either the opposite stereochemistry at any given chiral center or mixtures thereof are also envisioned. When chiral centers are found in the derivatives of this invention, it is to be understood that the compounds encompass all possible stereoisomers.

[0053] As discussed above, WM is a biologically unique low-grade B-cell lymphoma. WM is characterized by the presence of lymphoplasmacytic cells in the bone marrow and the secretion of IgM monoclonal protein in the serum, indicating that WM cells present a high protein turnover. Protein metabolism is a tightly regulated process, and inhibition of its turnover may lead to apoptosis in malignant cells, such as with proteasome inhibitors. The major activity of proteasome inhibitors is through targeting the IL-6 and NF-κB signaling pathways. Both these pathways are critical regulators of survival and proliferation in B-cell malignancies including WM.

[0054] The multicatalytic ubiquitin-proteasome pathway is responsible for the degradation of many eukaryotic cellular proteins. This pathway also controls the activation of NF-κB by regulating the degradation of IκBα. NF-κB plays a critical role in regulating many cellular responses including immunity, inflammation, proliferation, survival, and angiogenesis. Inactive NF-κB complexes with its inhibitor, IκBα, and remains sequestered in the cytosol. A variety of stimuli trigger the phosphorylation of IκB by IκB kinase (IKK). Phosphorylated IκB is then a target for ubiquitination and proteasome mediated degradation, which in turn releases NF-κB to translocate from the cytosol to the nucleus. Once in the nucleus, NF-κB stimulates transcription of numerous cytokines, chemokines, and cell adhesion molecules. NF-κB is constitutively activated in numerous hematologic malignancies, including plasma cell dyscrasias like multiple myeloma. This pathway also interacts with the PI3K/Akt pathway, a critical regulator of survival in WM cells based on our previous studies. Akt indirectly activates NF-κB through direct phosphorylation and activation of IκB kinase alpha (IKKα), thereby inducing degradation of NF-κB inhibitor alpha (IκBα) by the ubiquitin-proteasome pathway.

[0055] Bortezomib (Velcade, PS-341; Millennium Inc, Cambridge, Mass.), a proteasome inhibitor, inhibits the ubiquitin-26S proteasome pathway, which regulates the turnover of a vast number of intracellular proteins, has become an exciting target in a variety of malignancies, most notably multiple myeloma and Mantle Cell Lymphoma (MCL). The proper functioning of this system is crucial for cell cycle regulation, gene transcription, and signal transduction. Inhibition of the proteasome effectively decreases the degradation of I κ B α and prevents NF- κ B release and subsequent translocation to the nucleus. Based on its activity in multiple myeloma and MCL, single agent bortezomib was tested in WM in phase I trials and achieved 40-80% responses.

[0056] It has been shown that certain compounds described herein lead to inhibition of proliferation and induction of apoptosis in WM cell lines and CD19+ primary WM cells at doses achievable in vivo. In addition, the combination of certain compounds described herein and bortezomib leads to synergistic cytotoxicity on WM cell lines, IgM secreting cell lines and patient cells. These two agents lead to inhibition of nuclear translocation of p65 NF- κ B, with activity on the canonical and non-canonical NF- κ B pathway, and synergistic induction of caspases 3, 8 and 9 cleavage as well as PARP cleavage and induction of Smac/DIABLO.

[0057] Accordingly, some embodiments include administering a compound disclosed herein (e.g., a compound of Formula I) to a subject to treat or prevent WM. In some embodiments, the compound administered is salinosporamide A, described below. In some embodiments, a compound described herein is administered in combination with bortezomib to treat or prevent WM.

[0058] While not being bound by any particular theory, it is believed that the compounds described herein and bortezomib act synergistically through: the differential activity on the Akt pathway; and the differential activity on chymotrypsin-like, caspase-like and trypsin-like activities of the 20S proteasome. Certain compounds described herein induced cytotoxicity was completely abrogated in an Akt knockdown cell line, indicating that their major activity is mediated through the Akt pathway, while bortezomib modestly activated Akt activity. While not being bound by any particular theory, a major activity of the compounds described herein is mediated through regulation of Akt and therefore, their combination with bortezomib may overcome resistance to bortezomib in vivo.

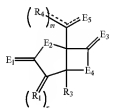
[0059] Certain compounds described herein and bortezomib inhibit migration and adhesion of WM cells to fibronectin present in the BM microenvironment. Adhesion of WM cells to fibronectin led to NF- κ B stimulation, which was abrogated by the compounds and bortezomib. In addition, the combination of these compounds and bortezomib led to inhibition of homing of WM cells in vivo in our homing model. Since IL-6 and NF- κ B induction by adhesion are two major pathways regulated by the proteasome, it is believed that the compounds described herein and bortezomib overcome resistance induced by mesenchymal cells and the addition of IL-6 in a co-culture in vitro system. Finally, the combination of bortezomib and certain compounds disclosed herein does not induce cytotoxicity on hematopoietic stem cells using colony-formation assays.

[0060] The adhesion of WM cells to the bone marrow milieu induces NF- κ B activation and IL-6 induces Akt activation, which were both down-regulated in the presence of certain compounds described herein either alone, and more

significantly in combination with bortezomib. The combination of the two agents overcomes the protective effect of the bone marrow niches, without affecting the growth and differentiation of normal hematopoietic components. In addition, homing is a complex process that is regulated by migration and adhesion of malignant cells to their specific bone marrow niches. It is believed that the compounds described herein and bortezomib inhibit migration and adhesion of WM cells as well as their homing in vivo.

Compounds of Formula I

[0061] In some embodiments, compounds for use as described herein are represented by Formula I:



Formula I

[0062] In certain embodiments the substituent(s) R_1 , R_3 , and R_4 separately may include a hydrogen, a halogen, a mono-substituted or a poly-substituted or an unsubstituted variant of the following residues: saturated C_1 - C_{24} alkyl, unsaturated C_2 - C_{24} alkenyl or C_2 - C_{24} alkynyl, acyl, acyloxy, alkylloxycarbonyloxy, aryloxy, carbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy carbonyl, alkoxy carbonyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkyl, hydroxy, alkylthio, arylthio, oxyalkyl, carboxy, cyano, thio, sulfoxide, sulfone, sulfonate esters, thiocyanate, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl. Further, in certain embodiments, each of E_1 , E_2 , E_3 , and E_4 can be a substituted or unsubstituted heteroatom, for example, a heteroatom or substituted heteroatom selected from the group consisting of nitrogen, sulfur and oxygen.

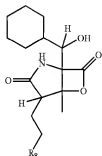
[0063] In some embodiments n can be equal to 1, while in others it can be equal to 2. When n is equal to 2, the substituents can be the same or can be different. Furthermore, in some embodiments R_3 is not a hydrogen. m can be equal to 1 or 2, and when m is equal to 2, R_4 can be the same or different.

[0064] E_5 can be, for example, OH, O, OR $_{10}$, S, SR $_{11}$, SO $_{2$ R $_{11}}$, NH, NH $_{2}$, NOH, NHOR $_{12}$, and NHOR $_{13}$, wherein R $_{10}$, R $_{11}$, and R $_{12}$ may separately include, for example, hydrogen, a substituted or unsubstituted of any of the following: alkyl, an aryl, a heteroaryl, and the like. Also, R $_1$ can be CH $_2$ CH $_2$ X, wherein X can be, for example, H, F, Cl, Br, and I. R $_3$ can be methyl. Furthermore, R $_4$ may include a cyclohexyl. Also, each of E_1 , E_3 , and E_4 can be O and E_2 can be NH. Preferably, R $_1$ can be CH $_2$ CH $_2$ X, wherein X is selected from the group consisting of H, F, Cl, Br, and I; wherein R $_4$ may include a cyclohexyl; wherein R $_3$ can be methyl; and wherein each of E_1 , E_3 , and E_4 separately can be O and E_2 can be NH.

[0065] In some embodiments, R $_2$ is not cyclohex-2-enyl carbinol when one of the R $_1$ substituents is ethyl or chloroethyl and R $_3$ is methyl.

[0066] In some embodiments, preferably R_1 is a substituted or unsubstituted C_1 to C_5 alkyl. For example, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, and pentyl are preferred. In some embodiments, R_1 is not a substituted or unsubstituted, unbranched C_6 alkyl.

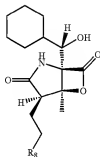
[0067] For example, an exemplary compound of Formula I has the following structure I-1:



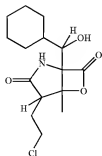
Formula I-1

[0068] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0069] Exemplary stereochemistry can be as follows:

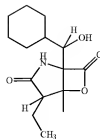


[0070] In preferred embodiments, the compound of Formula I has any of the following structures:

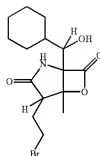


Formula I-2

-continued

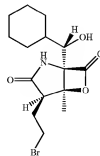
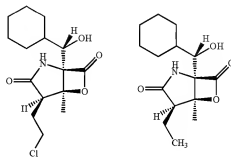


Formula I-3

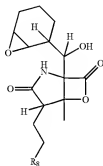


Formula I-4

[0071] The following is exemplary stereochemistry for compounds having the structures I-2, I-3, and I-4, respectively:



[0072] In other embodiments wherein R_4 may include a 7-oxa-bicyclo[4.1.0]hept-2-yl). An exemplary compound of Formula I is the following structure I-5:

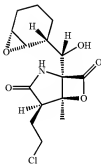


Formula I-5

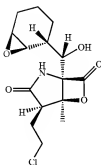
[0073] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0074] The following are examples of compounds having the structure of Formula I-5:

FORMULAE I-5A AND I-5B

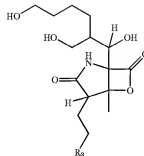


II-5A



II-5B

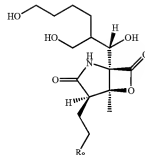
[0075] In still further embodiments, at least one R_4 may include a substituted or an unsubstituted branched alkyl. For example, a compound of Formula I can be the following structure I-6:



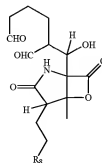
Formula I-6

[0076] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0077] The following is exemplary stereochemistry for a compound having the structure of Formula I-6:



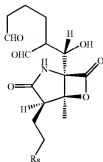
[0078] As another example, the compound of Formula I can be the following structure I-7:



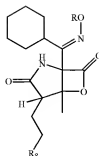
Formula I-7

[0079] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0080] The following is exemplary stereochemistry for a compound having the structure of Formula 1-7:

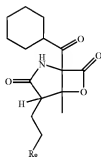


Formula 1-9



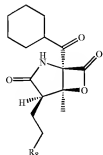
[0081] In other embodiments, at least one R_8 can be a cycloalkyl and E_5 can be an oxygen. An exemplary compound of Formula I can be the following structure 1-8:

Formula 1-8



[0082] R_8 may include, for example, hydrogen (1-8A), fluorine (1-8B), chlorine (1-8C), bromine (1-8D) and iodine (1-8E).

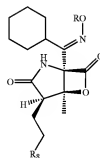
[0083] The following is exemplary stereochemistry for a compound having the structure of Formula 1-8:



[0084] In some embodiments E_5 can be an amine oxide, giving rise to an oxime. An exemplary compound of Formula I has the following structure 1-9:

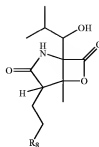
[0085] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine; R can be hydrogen, and a substituted or unsubstituted alkyl, aryl, or heteroaryl, and the like.

[0086] The following is exemplary stereochemistry for a compound having the structure of Formula 1-9:



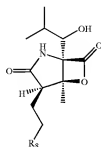
[0087] A further exemplary compound of Formula I has the following structure 1-10:

Formula 1-10

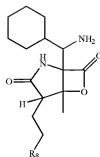


[0088] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

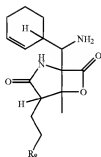
[0089] The following is exemplary stereochemistry for a compound having the structure of Formula 1-10:



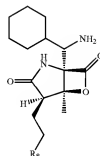
Formula 1-10



[0090] In some embodiments, E₅ can be NH₂. An exemplary compound of Formula I has the following structure 1-11:

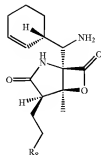


Formula 1-11

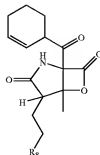


[0091] R₈ may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0092] The following is exemplary stereochemistry for a compound having the structure of Formula 1-11:



Formula 1-13



[0093] In some embodiments, at least one R₄ may include a cycloalkyl and E₅ can be NH₂. An exemplary compound of Formula I has the following structure 1-12:

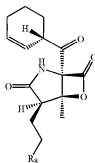
[0094] R₈ may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0095] The following is exemplary stereochemistry for a compound having the structure of Formula 1-12:

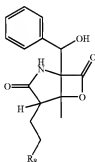
[0096] A further exemplary compound of Formula I has the following structure 1-13:

[0097] R₈ may include, for example, hydrogen (1-13A), fluorine (1-13B), chlorine (1-13C), bromine (1-13D) and iodine (1-13E).

[0098] The following is exemplary stereochemistry for a compound having the structure of Formula 1-13:



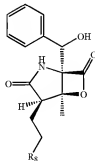
[0099] A still further exemplary compound of Formula I has the following structure 1-14:



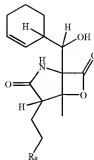
FORMULA 1-14

[0100] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0101] The following is exemplary stereochemistry for a compound having the structure of Formula 1-14:



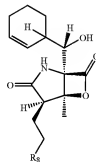
[0102] In some embodiments, the compounds of Formula I, may include as R_4 at least one cycloalkene, for example. Furthermore, in some embodiments, the compounds may include a hydroxy at E_5 , for example. A further exemplary compound of Formula I has the following structure 1-15:



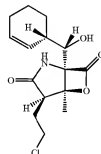
Formula 1-15

[0103] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

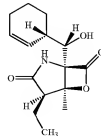
[0104] Exemplary stereochemistry can be as follows:



[0105] The following is exemplary stereochemistry for compounds having the structures 1-16, 1-17, 1-18, and 1-19, respectively:

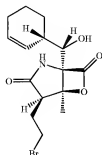


1-16

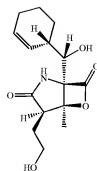


1-17

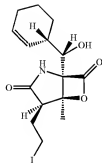
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I-18

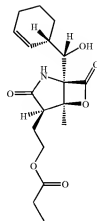


I-21



I-19

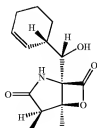
[0109] In some embodiments, the hydroxyl group of Formula I-21 can be esterified such that R_1 may include ethylpropionate, for example. An exemplary compound, Formula I-22, has the following structure and stereochemistry:



I-22

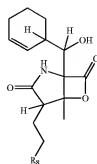
[0106] The compound of Formula I-16 is also known as salinosporamide A. The compounds of Formulae I-16, I-17, I-18 and I-19 can be obtained by fermentation, synthesis, or semi-synthesis and isolated/purified as set forth below. Furthermore, the compounds of Formulae I-16, I-17, I-18 and I-19 can be used, and are referred to, as “starting materials” to make other compounds described herein.

[0107] In some embodiments, the compounds of Formula I, may include a methyl group as R_1 , for example. A further exemplary compound, Formula I-20, has the following structure and stereochemistry:



I-20

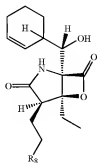
[0110] In some embodiments, the compounds of Formula I may include an ethyl group as R_1 , for example. A further exemplary compound of Formula I has the following structure I-23:



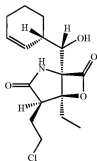
I-23

[0108] In some embodiments, the compounds of Formula I, may include hydroxyethyl as R_1 , for example. A further exemplary compound, Formula I-21, has the following structure and stereochemistry:

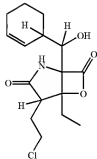
[0111] R_4 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine. Exemplary stereochemistry can be as follows:



[0112] In some embodiments, the compounds of Formula I-23 may have the following structure and stereochemistry, exemplified by Formula I-24C, where R_8 is chlorine:

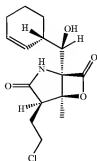


I-24C



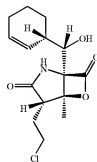
I-24C

[0113] In some embodiments, the compounds of Formula I-15 may have the following stereochemistry, exemplified by the compound of Formula I-25, where R_8 is chlorine:



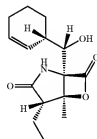
I-25

[0114] In some embodiments, the compound of Formula I-15 may have the following stereochemistry, exemplified by the compound of Formula I-26, where R_8 is chlorine:



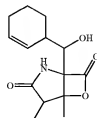
I-26

[0115] In some embodiments, the compound of Formula I may have the following structure and stereochemistry, exemplified by Formula I-27, where R_1 is ethyl:

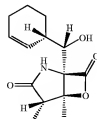


I-27

[0116] In some embodiments, the compound of Formula I may have the following structure and stereochemistry, exemplified by Formula I-28, where R_1 is methyl:

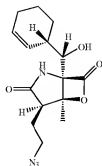


I-28



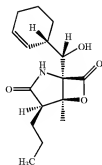
I-28

[0117] In some embodiments, the compounds of Formula I may include azidoethyl as R_1 , for example. A further exemplary compound, Formula I-29, has the following structure and stereochemistry:



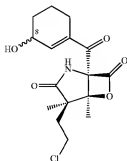
I-29

[0118] In some embodiments, the compounds of Formula I may include propyl as R_1 , for example. A further exemplary compound, Formula I-30, has the following structure and stereochemistry:



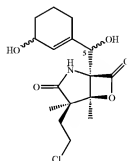
I-30

[0119] Still further exemplary compounds, Formulae I-31 and I-32, have the following structure and stereochemistry:



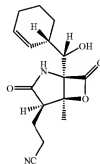
Formula I-31 and I-32

[0120] Other exemplary compounds, Formulae I-33, I-34, I-35 and I-36, have the following structure and stereochemistry:



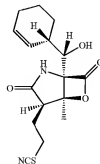
Formula I-33 and I-36

[0121] In some embodiments, the compound of Formula I may include cyanoethyl as R_1 ; for example, the compound of Formula I-37 has the following structure and stereochemistry:



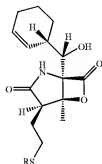
I-37

[0122] In another embodiment, the compound of Formula I may include ethylthiocyanate as R_1 ; for example, the compound of Formula I-38 has the following structure and stereochemistry:



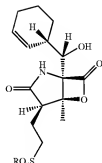
I-38

[0123] In some embodiments, the compounds of Formula I may include a thiol as R_1 , for example. A further exemplary compound, Formula I-39, has the following structure and stereochemistry, where $R=H$, alkyl, aryl, or substituted alkyl or aryl:



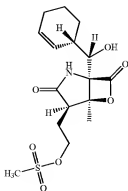
I-39

[0124] In a further exemplary compound, the sulfur of the compound of Formula I-39 can be oxidized to a sulfoxide ($n=1$) or sulfone ($n=2$), for example, as in the compound of Formula I-40:



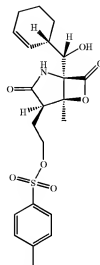
I-40

[0125] In some embodiments, the substituent R_1 of the compound of Formula I may include a leaving group, for example, a halogen, as in compounds I-18 or I-19, or another leaving group, such as a sulfonate ester. One example is the methane sulfonate (mesylate) of Formulae I-41A:



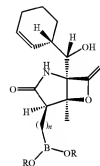
I-41A

[0126] Another embodiment is the tosylate of Formula I-41B



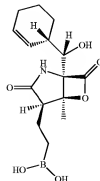
I-41B

[0127] In some embodiments, the substituent R_1 of the compound of Formula I may include electron acceptors. The electron acceptor can be, for example, a Lewis acid, such as a boronic acid or ester. An exemplary compound, Formula I-42, has the following structure and stereochemistry, where $n=0$, 1, 2, 3, 4, 5, or 6, for example, and where $R=H$ or alkyl, for example:

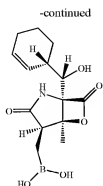


I-42

[0128] Further exemplary compounds of Formula I-42 are the compounds of Formula I-42A, where $n=2$ and $R=H$, and the compound of Formula I-42B, where $n=1$ and $R=H$:

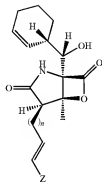


I-42A



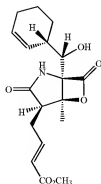
I-42B

[0129] In some embodiments where the substituent R_1 of the compound of Formula I includes an electron acceptor, the electron acceptor can be, for example, a Michael acceptor. An exemplary compound, Formula I-43 has the following structure, where $n=0$, 1, 2, 3, 4, 5, 6, and where Z is an electron withdrawing group, for example, CHO, COR, COOR, CONH_2 , CN, NO_2 , SOR, SO_2R , etc:



I-43

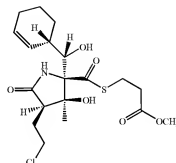
[0130] A further exemplary compound of Formula I-43 is the compound of Formula I-43A, where $n=1$ and $\text{Z}=\text{CO}_2\text{CH}_3$:



I-43A

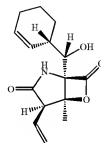
[0131] In some embodiments, the compounds can be prodrug esters or thioesters of the compounds of Formula I. For

example, the compound of Formula I-44 (a prodrug thioester of the compound of Formula I-16) has the following structure and stereochemistry:



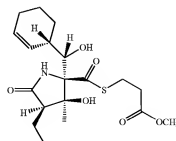
I-44

[0132] In some embodiments, the compounds of Formula I may include an alkenyl group as R_1 , for example, ethylenyl. A further exemplary compound, Formula I-46, has the following structure and stereochemistry:



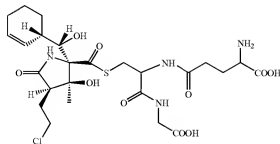
I-46

[0133] In some embodiments, the compounds can be prodrug esters or thioesters of the compounds of Formula I. For example, the compound of Formula I-47 (a prodrug thioester of the compound of Formula I-17) has the following structure and stereochemistry:

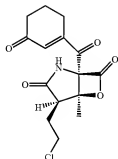


I-47

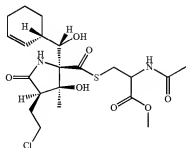
[0134] In some embodiments, the compounds can be prodrug esters or thioesters of the compounds of Formula I. For example, the compound of Formula I-48 has the following structure and stereochemistry:



[0135] Other exemplary compound, Formula 1-49 has the following structure and stereochemistry:



[0136] In some embodiments, the compound can be pro-drug ester or thioester of the compounds of Formula I. For example, the compound of Formula 1-50 (prodrug ester of the compound of Formula 1-16) has the following structure and stereochemistry:



[0137] Certain embodiments also provide pharmaceutically acceptable salts and pro-drug esters or thioesters of the compound of Formulae I, and provide methods of obtaining and purifying such compounds by the methods disclosed herein.

[0138] The term "pro-drug ester," especially when referring to a pro-drug ester of the compound of Formula I synthesized by the methods disclosed herein, refers to a chemical derivative of the compound that is rapidly transformed in vivo to yield the compound, for example, by hydrolysis in blood or

inside tissues. The term "pro-drug ester" refers to derivatives of the compounds disclosed herein formed by the addition of one of several ester- or thioester-forming groups that are hydrolyzed under physiological conditions. Examples of pro-drug ester groups include pivoxylomethyl, acetoxymethyl, phthalidyl, indanyl and methoxymethyl, as well as other such groups known in the art, including a (5-R-2-oxo-1,3-dioxole-4-yl)methyl group. Other prodrugs can be prepared by preparing a corresponding thioester of the compound, for example, by reacting with an appropriate thiol, such as thiophenol, Cysteine or derivatives thereof, or propanethiol, for example. Other examples of pro-drug ester groups can be found in, for example, T. Higuchi and V. Stella, in "Prodrugs as Novel Delivery Systems", Vol. 14, A.C.S. Symposium Series, American Chemical Society (1975); and "Bioreversible Carriers in Drug Design: Theory and Application", edited by E. B. Roche, Pergamon Press: New York, 14-21 (1987) (providing examples of esters useful as prodrugs for compounds containing carboxylic groups). Each of the above-mentioned references is hereby incorporated by reference in its entirety.

[0139] The term "pharmaceutically acceptable salt," as used herein, and particularly when referring to a pharmaceutically acceptable salt of a compound, including Formulae I, and Formula I as produced and synthesized by the methods disclosed herein, refers to any pharmaceutically acceptable salts of a compound, and preferably refers to an acid addition salt of a compound. Preferred examples of pharmaceutically acceptable salt are the alkali metal salts (sodium or potassium), the alkaline earth metal salts (calcium or magnesium), or ammonium salts derived from ammonia or from pharmaceutically acceptable organic amines, for example C_1 - C_7 alkylamine, cyclohexylamine, triethanolamine, ethylenediamine or tris-(hydroxymethyl)-aminomethane. With respect to compounds synthesized by the method of this embodiment that are basic amines, the preferred examples of pharmaceutically acceptable salts are acid addition salts of pharmaceutically acceptable inorganic or organic acids, for example, hydrohalic, sulfuric, phosphoric acid or aliphatic or aromatic carboxylic or sulfonic acid, for example acetic, succinic, lactic, malic, tartaric, citric, ascorbic, nicotinic, methanesulfonic, p-toluenesulfonic or naphthalenesulfonic acid.

[0140] Preferred pharmaceutical compositions disclosed herein include pharmaceutically acceptable salts and pro-drug esters of the compound of Formulae I obtained and purified by the methods disclosed herein. Accordingly, if the manufacture of pharmaceutical formulations involves intimate mixing of the pharmaceutical excipients and the active ingredient in its salt form, then it is preferred to use pharmaceutical excipients which are non-basic, that is, either acidic or neutral excipients.

[0141] It will be also appreciated that the phrase "compounds and compositions comprising the compound," or any like phrase, is meant to encompass compounds in any suitable form for pharmaceutical delivery, as discussed in further detail herein. For example, in certain embodiments, the compounds or compositions comprising the same may include a pharmaceutically acceptable salt of the compound.

[0142] The term "halogen atom," as used herein, means any one of the radio-stable atoms of column 7 of the Periodic Table of the Elements, i.e., fluorine, chlorine, bromine, or iodine, with bromine and chlorine being preferred.

[0143] The term "alkyl," as used herein, means any unbranched or branched, substituted or unsubstituted, satu-

rated hydrocarbon, with C_1 - C_{24} preferred, and C_1 - C_6 hydrocarbons being preferred, with methyl, ethyl, propyl, isopropyl, butyl, isobutyl, and tert-butyl, and pentyl being most preferred. Among the substituted, saturated hydrocarbons, C_1 - C_{24} are preferred, with C_1 - C_6 mono- and di- and per-halogen substituted saturated hydrocarbons and amino-substituted hydrocarbons more preferred.

[0144] The term "substituted" has its ordinary meaning, as found in numerous contemporary patents from the related art. See, for example, U.S. Pat. Nos. 6,509,331; 6,506,787; 6,500,825; 5,922,683; 5,886,210; 5,874,443; and 6,350,759; all of which are incorporated herein in their entireties by reference. Specifically, the definition of substituted is as broad as that provided in U.S. Pat. No. 6,509,331, which defines the term "substituted alkyl" such that it refers to an alkyl group, preferably of from 1 to 10 carbon atoms, having from 1 to 5 substituents, and preferably 1 to 3 substituents, selected from the group consisting of alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, acyl, acylamino, acyloxy, amino, substituted amino, aminoacyl, aminoacyloxy, oxyacylamino, cyano, halogen, hydroxyl, carboxyl, carboxylalkyl, keto, thioketo, thiol, thioalkoxy, substituted thioalkoxy, thiocyanate, aryl, arylalkoxy, heteroaryl, heteroaryloxy, heterocyclic, heterocycloxy, hydroxyamino, alkoxyamino, nitro, azido, boronic acid, boronic ester —SO-alkyl, —SO-substituted alkyl, —SO-aryl, —SO-heteroaryl, —SO₂-alkyl, —SO₂-substituted alkyl, —SO₂-aryl, —SO₂-heteroaryl, —OSO-alkyl, —OSO-substituted alkyl, —OSO-aryl, —OSO-heteroaryl, —OSO₂-alkyl, —OSO₂-substituted alkyl, —OSO₂-aryl, and —OSO₂-heteroaryl. The other above-listed patents also provide standard definitions for the term "substituted" that are well-understood by those of skill in the art.

[0145] The term "cycloalkyl" refers to any non-aromatic hydrocarbon ring, preferably having five to twelve atoms comprising the ring. The term "acyl" refers to alkyl or aryl groups derived from an oxoacid, with an acetyl group being preferred.

[0146] The term "alkenyl," as used herein, means any unbranched or branched, substituted or unsubstituted, unsaturated hydrocarbon including polyunsaturated hydrocarbons, with C_1 - C_6 unbranched, mono-unsaturated and di-unsaturated, unsubstituted hydrocarbons being preferred, and mono-unsaturated, di-halogen substituted hydrocarbons being most preferred. The term "cycloalkenyl" refers to any non-aromatic hydrocarbon ring, preferably having five to twelve atoms comprising the ring.

[0147] The terms "aryl," "substituted aryl," "heteroaryl," and "substituted heteroaryl," as used herein, refer to aromatic hydrocarbon rings, preferably having five, six, or seven atoms, and most preferably having six atoms comprising the ring. "Heteroaryl" and "substituted heteroaryl," refer to aromatic hydrocarbon rings in which at least one heteroatom, e.g. oxygen, sulfur, or nitrogen atom, is in the ring along with at least one carbon atom. The term "heterocycle" or "heterocyclic" refer to any cyclic compound containing one or more heteroatoms. The substituted aryls, heterocycles and heteroaryls can be substituted with any substituent, including those described above and those known in the art.

[0148] The term "alkoxy" refers to any unbranched, or branched, substituted or unsubstituted, saturated or unsaturated ether, with C_1 - C_6 unbranched, saturated, unsubstituted ethers being preferred, with methoxy being preferred, and also with dimethyl, diethyl, methyl-isobutyl, and methyl-tert-

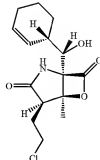
butyl ethers also being preferred. The term "cycloalkoxy" refers to any non-aromatic hydrocarbon ring, preferably having five to twelve atoms comprising the ring. The term "alkoxy carbonyl" refers to any linear, branched, cyclic, saturated, unsaturated, aliphatic or aromatic alkoxy attached to a carbonyl group. The examples include methoxycarbonyl group, ethoxycarbonyl group, propoxycarbonyl group, isopropoxycarbonyl group, butoxycarbonyl group, sec-butoxycarbonyl group, tert-butoxycarbonyl group, cyclopentoxycarbonyl group, cyclohexoxycarbonyl group, benzoyloxycarbonyl group, allyloxycarbonyl group, phenyloxycarbonyl group, pyridyloxycarbonyl group, and the like.

[0149] The terms "pure," "purified," "substantially purified," and "isolated" as used herein refer to the compound of the embodiment being free of other, dissimilar compounds with which the compound, if found in its natural state, would be associated in its natural state. In certain embodiments described as "pure," "purified," "substantially purified," or "isolated" herein, the compound may comprise at least 0.5%, 1%, 5%, 10%, or 20%, and most preferably at least 50% or 75% of the mass, by weight, of a given sample.

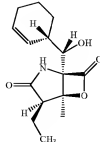
[0150] The terms "derivative," "variant," or other similar term refers to a compound that is an analog of the other compound.

[0151] Certain of the compounds of Formula I can be obtained and purified or can be obtained via semi-synthesis from purified compounds as set forth herein. Generally, without being limited thereto, the compounds of Formula I-15, preferably, Formulae I-16, I-17, I-18 and I-19, can be obtained synthetically or by fermentation. Exemplary fermentation procedures are provided below. Further, the compounds of Formula I-15, preferably, Formulae I-16, I-17, I-18 and I-19 can be used as starting compounds in order to obtain/synthesize various of the other compounds described herein. Exemplary non-limiting syntheses are provided herein.

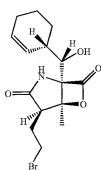
Formula I-16



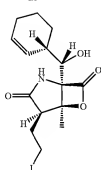
Formula I-17



-continued



Formula 1-18



Formula 1-19

[0152] Formula 1-16 may be produced through a high-yield saline fermentation (~350-400 mg/L) and modifications of the conditions have yielded new analogs in the fermentation extracts. Additional analogs can be generated through directed biosynthesis. Directed biosynthesis is the modification of a natural product by adding biosynthetic precursor analogs to the fermentation of producing microorganisms (Lam, et al., *J Antibiot* (Tokyo) 44:934 (1991), Lam, et al., *J Antibiot* (Tokyo) 54:1 (2001), which is hereby incorporated by reference in its entirety).

[0153] Exposing the producing culture to analogs of acetic acid, phenylalanine, valine, butyric acid, shikimic acid, and halogens, preferably, other than chlorine, can lead to the formation of new analogs. The new analogs produced can be easily detected in crude extracts by HPLC and LC-MS. For example, after manipulating the medium with different concentrations of sodium bromide, a bromo-analog, Formula 1-18, was successfully produced in shake-flask culture at a titer of 14 mg/L.

[0154] A second approach to generate analogs is through biotransformation. Biotransformation reactions are chemical reactions catalyzed by enzymes or whole cells containing these enzymes. Zaks, A., *Curr Opin Chem Biol* 5:130 (2001). Microbial natural products are ideal substrates for biotransformation reactions as they are synthesized by a series of enzymatic reactions inside microbial cells. Riva, S., *Curr Opin Chem Biol* 5:106 (2001).

[0155] Given the structure of the described compounds, including those of Formula 1-15, for example, the possible biosynthetic origins are acetyl-CoA, ethylmalonyl-CoA, phenylalanine and chorine. Ethylmalonyl-CoA is derived from butyryl-CoA, which can be derived either from valine or crotonyl-CoA. Liu, et al., *Metab Eng* 3:40 (2001). Phenylalanine is derived from shikimic acid.

[0156] Alternatively, compositions such as Formula 1-16 and its analogs may be produced synthetically, e.g., such as described in U.S. application Ser. No. 11/697,689, which is incorporated by reference in its entirety.

Production of Compounds of Formulae 1-16, 1-17, 1-18, 1-20, 1-24C, 1-26, 1-27 and 1-28

[0157] The production of compounds of Formulae 1-16, 1-17, 1-18, 1-20, 1-24C, 1-26, 1-27 and 1-28 can be carried out by cultivating strain CNB476 and strain NPS21184, a natural variant of strain CNB476, in a suitable nutrient medium under conditions described herein, preferably under submerged aerobic conditions, until a substantial amount of compounds are detected in the fermentation; harvesting by extracting the active components from the fermentation broth with a suitable solvent; concentrating the solvent containing the desired components; then subjecting the concentrated material to chromatographic separation to isolate the compounds from other metabolites also present in the cultivation medium.

[0158] The culture (CNB476) was deposited on Jun. 20, 2003 with the American Type Culture Collection (ATCC) in Rockville, Md. and assigned the ATCC patent deposition number PTA-5275. Strain NPS21184, a natural variant of strain CNB476, was derived from strain CNB476 as a single colony isolate. Strain NPS21184 has been deposited to ATCC on Apr. 27, 2005. The ATCC deposit meets all of the requirements of the Budapest treaty. The culture is also maintained at and available from Nereus Pharmaceutical Culture Collection at 10480 Wateridge Circle, San Diego, Calif. 92121. In addition to the specific microorganism described herein, it should be understood that mutants, such as those produced by the use of chemical or physical mutagens including X-rays, etc. and organisms whose genetic makeup has been modified by molecular biology techniques, may also be cultivated to produce the starting compounds of Formulae 1-16, 1-17, and 1-18.

Fermentation of Strain CNB476 and Strain NPS21184

[0159] Production of compounds can be achieved at temperature conducive to satisfactory growth of the producing organism, e.g. from 16 degree C. to 40 degree C., but it is preferable to conduct the fermentation at 22 degree C. to 32 degree C. The aqueous medium can be incubated for a period of time necessary to complete the production of compounds as monitored by high pressure liquid chromatography (HPLC), preferably for a period of about 2 to 10 days, on a rotary shaker operating at about 50 rpm to 400 rpm, preferably at 150 rpm to 250 rpm, for example. The production of the compounds can also be achieved by cultivating the production strain in a bioreactor, such as a fermenter system that is suitable for the growth of the production strain.

[0160] Growth of the microorganisms can be achieved by one of ordinary skill of the art by the use of appropriate medium. Broadly, the sources of carbon include glucose, fructose, mannose, maltose, galactose, mannitol and glycerol, other sugars and sugar alcohols, starches and other carbohydrates, or carbohydrate derivatives such as dextran, cerelose, as well as complex nutrients such as oat flour, corn meal, millet, corn, and the like. The exact quantity of the carbon source that is utilized in the medium will depend in part, upon the other ingredients in the medium, but an amount of carbohydrate between 0.5 to 25 percent by weight of the medium can be satisfactorily used, for example. These carbon

sources can be used individually or several such carbon sources can be combined in the same medium, for example. Certain carbon sources are preferred as hereinafter set forth.

[0161] The sources of nitrogen include amino acids such as glycine, arginine, threonine, methionine and the like, ammonium salt, as well as complex sources such as yeast extracts, corn steep liquors, distiller solubles, soybean meal, cottonseed meal, fish meal, peptone, and the like. The various sources of nitrogen can be used alone or in combination in amounts ranging from 0.5 to 25 percent by weight of the medium, for example.

[0162] Among the nutrient inorganic salts, which can be incorporated in the culture media, are the customary salts capable of yielding sodium, potassium, magnesium, calcium, phosphate, sulfate, chloride, carbonate, and like ions. Also included are trace metals such as cobalt, manganese, iron, molybdenum, zinc, cadmium, and the like. Pharmaceutical Compositions

[0163] In one embodiment, the compounds disclosed herein are used in pharmaceutical compositions. The compounds preferably can be produced by the methods disclosed herein. The compounds can be used, for example, in pharmaceutical compositions comprising a pharmaceutically acceptable carrier prepared for storage and subsequent administration. Also, embodiments relate to a pharmaceutically effective amount of the products and compounds disclosed above in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro ed. 1985), which is incorporated herein by reference in its entirety. Preservatives, stabilizers, dyes and even flavoring agents can be provided in the pharmaceutical composition. For example, sodium benzoate, ascorbic acid and esters of p-hydroxybenzoic acid can be added as preservatives. In addition, antioxidants and suspending agents can be used.

[0164] The compositions can be formulated and used as tablets, capsules, or elixirs for oral administration; suppositories for rectal administration; sterile solutions, suspensions for injectable administration; patches for transdermal administration, and sub-dermal deposits and the like. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (for example, liposomes), can be utilized.

[0165] Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or other organic oils such as soybean, grapefruit or almond oils, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the

suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[0166] Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents can be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions can be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyes/stuffs or pigments can be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. For this purpose, concentrated sugar solutions can be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyes/stuffs or pigments can be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. Such formulations can be made using methods known in the art (see, for example, U.S. Pat. No. 5,733,888 (injectable compositions); U.S. Pat. No. 5,726,181 (poorly water soluble compounds); U.S. Pat. No. 5,707,641 (therapeutically active proteins or peptides); U.S. Pat. No. 5,667,809 (lipophilic agents); U.S. Pat. No. 5,576,012 (solubilizing polymeric agents); U.S. Pat. No. 5,707,615 (anti-viral formulations); U.S. Pat. No. 5,683,676 (particulate medicaments); U.S. Pat. No. 5,654,286 (topical formulations); U.S. Pat. No. 5,688,529 (oral suspensions); U.S. Pat. No. 5,445,829 (extended release formulations); U.S. Pat. No. 5,653,987 (liquid formulations); U.S. Pat. No. 5,641,515 (controlled release formulations) and U.S. Pat. No. 5,601,845 (spheroid formulations); all of which are incorporated herein by reference in their entireties.

[0167] Further disclosed herein are various pharmaceutical compositions well known in the pharmaceutical art for uses that include topical, intraocular, intranasal, and intraauricular delivery. Pharmaceutical formulations include aqueous ophthalmic solutions of the active compounds in water-soluble form, such as eyedrops, or in gellan gum (Shedden et al., *Clin. Ther.*, 23(3):440-50 (2001)) or hydrogels (Mayer et al., *Ophthalmologica*, 210(2): 101-3 (1996)); ophthalmic ointments; ophthalmic suspensions, such as microparticles, drug-containing small polymeric particles that are suspended in a liquid carrier medium (Joshi, A. 1994 *J Ocul Pharmacol* 10:29-45); lipid-soluble formulations (Alm et al., *Prog. Clin. Biol. Res.*, 312:447-58 (1989)); and microspheres (Mordenti, *Toxicol. Sci.*, 52(1): 101-6 (1999)); and ocular inserts. All of the above-mentioned references, are incorporated herein by reference in their entireties. Such suitable pharmaceutical formulations are most often and preferably formulated to be sterile, isotonic and buffered for stability and comfort. Pharmaceutical compositions may also include drops and sprays

often prepared to simulate in many respects nasal secretions to ensure maintenance of normal ciliary action. As disclosed in Remington's Pharmaceutical Sciences (Mack Publishing, 18th Edition), which is incorporated herein by reference in its entirety, and well-known to those skilled in the art, suitable formulations are most often and preferably isotonic, slightly buffered to maintain a pH of 5.5 to 6.5, and most often and preferably include anti-microbial preservatives and appropriate drug stabilizers. Pharmaceutical formulations for intra-ocular delivery include suspensions and ointments for topical application in the eye. Common solvents for such ocular formulations include glycerin and water.

[0168] When used as an anti-cancer compound, for example, the compounds of Formulae I or compositions including Formulae I can be administered by either oral or non-oral pathways. When administered orally, it can be administered in capsule, tablet, granule, spray, syrup, or other such form. When administered non-orally, it can be administered as an aqueous suspension, an oily preparation or the like or as a drip, suppository, salve, ointment or the like, when administered via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, or the like.

[0169] In one embodiment, the anti-cancer agent can be mixed with additional substances to enhance their effectiveness.

Methods of Administration

[0170] In an alternative embodiment, the disclosed chemical compounds and the disclosed pharmaceutical compositions are administered by a particular method as an anti-microbial. Such methods include, among others, (a) administration through oral pathways, which administration includes administration in capsule, tablet, granule, spray, syrup, or other such forms; (b) administration through non-oral pathways, which administration includes administration as an aqueous suspension, an oily preparation or the like or as a drip, suppository, salve, ointment or the like; administration via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, intradermally, or the like; as well as (c) administration topically, (d) administration rectally, or (e) administration vaginally, as deemed appropriate by those of skill in the art for bringing the compound of the present embodiment into contact with living tissue; and (f) administration via controlled released formulations, depot formulations, and infusion pump delivery. As further examples of such modes of administration and as further disclosure of modes of administration, disclosed herein are various methods for administration of the disclosed chemical compounds and pharmaceutical compositions including modes of administration through intraocular, intranasal, and intraauricular pathways.

[0171] The pharmaceutically effective amount of the compositions that include the described compounds required as a dose will depend on the route of administration, the type of animal, including human, being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize.

[0172] In practicing the methods of the embodiment, the products or compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized in vivo,

ordinarily in a mammal, preferably in a human, or in vitro. In employing them in vivo, the products or compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, vaginally, nasally or intraperitoneally, employing a variety of dosage forms. Such methods may also be applied to testing chemical activity in vivo.

[0173] As will be readily apparent to one skilled in the art, the useful in vivo dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular compounds employed, and the specific use for which these compounds are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, can be accomplished by one skilled in the art using routine pharmacological methods. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. Alternatively, acceptable in vitro studies can be used to establish useful doses and routes of administration of the compositions identified by the present methods using established pharmacological methods.

[0174] In non-human animal studies, applications of potential products are commenced at higher dosage levels, with dosage being decreased until the desired effect is no longer achieved or adverse side effects disappear. The dosage may range broadly, depending upon the desired effects and the therapeutic indication. Typically, dosages can be between about 10 microgram/kg and 100 mg/kg body weight, preferably between about 100 microgram/kg and 10 mg/kg body weight. Alternatively dosages can be based and calculated upon the surface area of the patient, as understood by those of skill in the art. Administration is preferably oral on a daily or twice daily basis.

[0175] The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. See for example, Fingl et al., in *The Pharmacological Basis of Therapeutics*, 1975, which is incorporated herein by reference in its entirety. It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above can be used in veterinary medicine.

[0176] Depending on the specific conditions being treated, such agents can be formulated and administered systemically or locally. A variety of techniques for formulation and administration can be found in Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, Pa. (1990), which is incorporated herein by reference in its entirety. Suitable administration routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous,

intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

[0177] For injection, the agents of the embodiment can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For such transcutaneous administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the embodiment into dosages suitable for systemic administration is within the scope of the embodiment. With proper choice of carrier and suitable manufacturing practice, the compositions disclosed herein, in particular, those formulated as solutions, can be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the embodiment to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

[0178] Agents intended to be administered intracellularly can be administered using techniques well known to those of ordinary skill in the art. For example, such agents can be encapsulated into liposomes, then administered as described above. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external micro-environment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules can be directly administered intracellularly.

[0179] Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration can be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions can be manufactured in a manner that is itself known, for example, by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

[0180] Compounds disclosed herein can be evaluated for efficacy and toxicity using known methods. For example, the toxicology of a particular compound, or of a subset of the compounds, sharing certain chemical moieties, can be established by determining *in vitro* toxicity towards a cell line, such as a mammalian, and preferably human, cell line. The results of such studies are often predictive of toxicity in animals, such as mammals, or more specifically, humans. Alternatively, the toxicity of particular compounds in an animal model, such as mice, rats, rabbits, dogs or monkeys, can be determined using known methods. The efficacy of a particular compound can be established using several art recognized methods, such as *in vitro* methods, animal models, or human clinical trials. Art-recognized *in vitro* models exist for nearly every class of condition, including the conditions abated by

the compounds disclosed herein, including cancer, cardiovascular disease, and various immune dysfunction, and infectious diseases. Similarly, acceptable animal models can be used to establish efficacy of chemicals to treat such conditions. When selecting a model to determine efficacy, the skilled artisan can be guided by the state of the art to choose an appropriate model, dose, and route of administration, and regime. Of course, human clinical trials can also be used to determine the efficacy of a compound in humans.

[0181] When used as an anti-cancer agent, the compounds disclosed herein can be administered by either oral or a non-oral pathways. When administered orally, it can be administered in capsule, tablet, granule, spray, syrup, or other such form. When administered non-orally, it can be administered as an aqueous suspension, an oily preparation or the like or as a drip, suppository, salve, ointment or the like, when administered via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, intradermally, or the like. Controlled release formulations, depot formulations, and infusion pump delivery are similarly contemplated.

[0182] The compositions disclosed herein in pharmaceutical compositions may also comprise a pharmaceutically acceptable carrier. Such compositions can be prepared for storage and for subsequent administration. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). For example, such compositions can be formulated and used as tablets, capsules, or solutions for oral administration; suppositories for rectal or vaginal administration; sterile solutions or suspensions for injectable administration. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients include, but are not limited to, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (for example, liposomes), can be utilized.

[0183] The pharmaceutically effective amount of the composition required as a dose will depend on the route of administration, the type of animal being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize.

[0184] The products or compositions of the embodiment, as described above, can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized *in vivo* or *in vitro*. The useful dosages and the most useful modes of administration will vary depending upon the age, weight and animal treated, the particular compounds employed, and the specific use for which these composition or compositions are employed. The magnitude of a dose in the management or treatment for a particular disorder will vary with the severity of the condition to be treated and to the route of administration, and depending on the disease conditions and their severity, the compositions can be formulated and administered either systemically or locally. A variety of techniques for

formulation and administration can be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, Pa. (1990).

[0185] To formulate the compounds of Formulae I as an anti-cancer agent, known surface active agents, excipients, smoothing agents, suspension agents and pharmaceutically acceptable film-forming substances and coating assistants, and the like can be used. Preferably alcohols, esters, sulfated aliphatic alcohols, and the like can be used as surface active agents; sucrose, glucose, lactose, starch, crystallized cellulose, mannitol, light anhydrous silicate, magnesium aluminate, magnesium methasilicate aluminate, synthetic aluminum silicate, calcium carbonate, sodium acid carbonate, calcium hydrogen phosphate, calcium carboxymethyl cellulose, and the like can be used as excipients; magnesium stearate, talc, hardened oil and the like can be used as smoothing agents; coconut oil, olive oil, sesame oil, peanut oil, soya can be used as suspension agents or lubricants; cellulose acetate phthalate as a derivative of a carbohydrate such as cellulose or sugar, or methylacetate-methylacrylate copolymer as a derivative of polyvinyl can be used as suspension agents; and plasticizers such as ester phthalates and the like can be used as suspension agents. In addition to the foregoing preferred ingredients, sweeteners, fragrances, colorants, preservatives and the like can be added to the administered formulation of the compound produced by the method of the embodiment, particularly when the compound is to be administered orally.

[0186] The compounds and compositions can be orally or non-orally administered to a human patient in the amount of about 0.001 mg/kg/day to about 10,000 mg/kg/day of the active ingredient, and more preferably about 0.1 mg/kg/day to about 100 mg/kg/day of the active ingredient at, preferably, one time per day or, less preferably, over two to about ten times per day. Alternatively and also preferably, the compound produced by the method of the embodiment may preferably be administered in the stated amounts continuously by, for example, an intravenous drip. Thus, for the example of a patient weighing 70 kilograms, the preferred daily dose of the active ingredient would be about 0.07 mg/day to about 700 gm/day, and more preferably, 7 mg/day to about 7 grams/day. Nonetheless, as will be understood by those of skill in the art, in certain situations it can be necessary to administer the anti-cancer compound of the embodiment in amounts that excess, or even far exceed, the above-stated, preferred dosage range to effectively and aggressively treat particularly advanced cancers or infections.

[0187] In the case of using the cancer produced by methods of the embodiment as a biochemical test reagent, the compound produced by methods of the embodiment inhibits the progression of the disease when it is dissolved in an organic solvent or hydrous organic solvent and it is directly applied to any of various cultured cell systems. Usable organic solvents include, for example, methanol, methylsulfoxide, and the like. The formulation can, for example, be a powder, granular or other solid inhibitor, or a liquid inhibitor prepared using an organic solvent or a hydrous organic solvent. While a preferred concentration of the compound produced by the method of the embodiment for use as an anticancer compound is generally in the range of about 1 to about 100 µg/ml, the most appropriate use amount varies depending on the type of cultured cell system and the purpose of use, as will be appreciated by persons of ordinary skill in the art. Also, in

certain applications it can be necessary or preferred to persons of ordinary skill in the art to use an amount outside the foregoing range.

[0188] In one embodiment, the method of using a compound as an anti-cancer involves administering an effective amount of any of the compounds of Formulae I or compositions of those compounds. In a preferred embodiment, the method involves administering the compound represented by Formula I, to a patient in need of an anti-cancer agent, until the need is effectively reduced or more preferably removed.

[0189] As will be understood by one of skill in the art, "need" is not an absolute term and merely implies that the patient can benefit from the treatment of the anti-cancer agent in use. By "patient" what is meant is an organism that can benefit by the use of an anti-cancer agent. For example, any organism with cancer, such as, WM. In one embodiment, the patient's health may not require that an anti-cancer agent be administered, however, the patient may still obtain some benefit by the reduction of the level of cancer cells present in the patient, and thus be in need. In one embodiment, the anti-cancer agent is effective against one type of cancer, but not against other types; thus, allowing a high degree of selectivity in the treatment of the patient. In choosing such an anti-cancer agent, the methods and results disclosed in the Examples can be useful. In still further embodiments, the anti-cancer agent is effective against a broad spectrum of cancers or all cancers. Examples of cancers, against which the compounds can be effective include WM, a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma, multiple myelomas, a melanoma, and the like.

[0190] "Therapeutically effective amount," "pharmaceutically effective amount," or similar term, means that amount of drug or pharmaceutical agent that will result in a biological or medical response of a cell, tissue, system, animal, or human that is being sought. In a preferred embodiment, the medical response is one sought by a researcher, veterinarian, medical doctor, or other clinician.

[0191] "Anti-cancer agent" refers to a compound or composition including the compound that reduces the likelihood of survival of a cancer cell. In one embodiment, the likelihood of survival is determined as a function of an individual cancer cell; thus, the anti-cancer agent will increase the chance that an individual cancer cell will die. In one embodiment, the likelihood of survival is determined as a function of a population of cancer cells; thus, the anti-cancer agent will increase the chances that there will be a decrease in the population of cancer cells. In one embodiment, anti-cancer agent means chemotherapeutic agent or other similar term.

[0192] A "chemotherapeutic agent" is a chemical compound useful in the treatment of a neoplastic disease, such as cancer. Examples of chemotherapeutic agents include alkylating agents, such as a nitrogen mustard, an ethylenimine and a methylmelamine, an alkyl sulfonate, a nitrosourea, and a triazene, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, corticosteroids, a natural product such as a vinca alkaloid, an epipodophyllotoxin, an antibiotic, an enzyme, a taxane, and a biological response modifier or antibodies to biological response modifiers or other agents; miscellaneous agents such as a platinum coordination complex, an anthracenedione, an anthracycline, a substituted urea, a methyl hydrazine derivative, or an adrenocortical suppress-

sant; or a hormone or an antagonist such as an adrenocorticosteroid, a progestin, an estrogen, an antiestrogen, an androgen, an antiandrogen, or a gonadotropin-releasing hormone analog. Specific examples include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytoxan, Taxol, Toxotere, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincristine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Caminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins, Melphalan, and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

[0193] The anti-cancer agent may act directly upon a cancer cell to kill the cell, induce death of the cell, to prevent division of the cell, and the like. Alternatively, the anti-cancer agent may indirectly act upon the cancer cell by limiting nutrient or blood supply to the cell, for example. Such anti-cancer agents are capable of destroying or suppressing the growth or reproduction of cancer cells, such as a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma, multiple myelomas, a melanoma, and the like.

[0194] In one embodiment, a described compound, preferably a compound having the Formulae I, including those as described herein, is considered an effective anti-cancer agent if the compound can influence 10% of the cancer cells, for example. In a more preferred embodiment, the compound is effective if it can influence 10 to 50% of the cancer cells. In an even more preferred embodiment, the compound is effective if it can influence 50-80% of the cancer cells. In an even more preferred embodiment, the compound is effective if it can influence 80-95% of the cancer cells. In an even more preferred embodiment, the compound is effective if it can influence 95-99% of the cancer cells. "Influence" is defined by the mechanism of action for each compound. For example, if a compound prevents the division of cancer cells, then influence is a measure of prevention of cancer cell division. Not all mechanisms of action need be at the same percentage of effectiveness. In an alternative embodiment, a low percentage effectiveness can be desirable if the lower degree of effectiveness is offset by other factors, such as the specificity of the compound, for example. Thus a compound that is only 10% effective, for example, but displays little in the way of harmful side-effects to the host, or non-harmful microbes or cells, can still be considered effective.

[0195] In one embodiment, the compounds described herein are administered simply to remove cancer cells and need not be administered to a patient. For example, the compounds can be administered *ex vivo* to a cell sample, such as a bone marrow or stem cell transplant to ensure that only non-cancerous cells are introduced into the recipient. After the compounds are administered they may optionally be removed. Whether or not this is an option will depend upon the relative needs of the situation and the risks associated with the compound, which in part can be determined as described in the Examples below.

[0196] The following non-limiting examples are meant to describe the preferred embodiments of the methods. Variations in the details of the particular methods employed and in

the precise chemical compositions obtained will undoubtedly be appreciated by those of skill in the art.

EXAMPLES

Example 1

Fermentation of Compound of Formulae I-16, I-17, I-20, I-24C, I-26 and I-28 Using Strain CNB476

[0197] Strain CNB476 was grown in a 500-ml flask containing 100 ml of vegetative medium consisting of the following per liter of deionized water: glucose, 4 g; Bacto tryptone, 3 g; Bacto casitone, 5 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28 degree C. for 3 days on a rotary shaker operating at 250 rpm. Five ml each of the first seed culture was inoculated into three 500-ml flasks containing of 100 ml of the vegetative medium. The second seed cultures were incubated at 28 degree C. and 250 rpm on a rotary shaker for 2 days. Five ml each of the second seed culture was inoculated into thirty-five 500-ml flasks containing of 100 ml of the vegetative medium. The third seed cultures were incubated at 28 degree and 250 rpm on a rotary shaker for 2 days. Five ml each of the third seed culture was inoculated into four hundred 500-ml flasks containing 100 ml of the Production Medium A consisting of the following per liter of deionized water: starch, 10 g; yeast extract, 4 g; Hy-Soy, 4 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The production cultures were incubated at 28 degree C. and 250 rpm on rotary shakers for 1 day. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were added to the production cultures. The production cultures were further incubated at 28 degree C. and 250 rpm on rotary shakers for 5 days and achieved a titer of Compound I-16 of about 200 mg/L. The culture broth was filtered through cheese cloth to recover the Amberlite XAD-7 resin. The resin was extracted with 2 times 6 liters ethyl acetate followed by 1 time 1.5 liters ethyl acetate. The combined extracts were dried *in vacuo*. The dried extract, containing 3.8 grams the compound of Formula I-16 and lesser quantities of compounds of formulae I-20 and I-24C, was then processed for the recovery of the compounds of Formula I-16, I-20, I-24C, I-26 and I-28.

Example 2

Fermentation of Compounds I-16, I-17, I-20, I-24C, I-26 and I-28 Using Strain NPS21184

[0198] Strain NPS21184 was grown in a 500-ml flask containing 100 ml of vegetative medium consisting of the following per liter of deionized water: glucose, 8 g; yeast extract, 6 g; Hy-Soy, 6 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28 degree C. for 3 days on a rotary shaker operating at 250 rpm. Five ml of the first seed culture was inoculated into 500-ml flask containing of 100 ml of the vegetative medium. The second seed cultures were incubated at 28 degree C. and 250 rpm on a rotary shaker for 2 days. Five ml each of the second seed culture was inoculated into 500-ml flask containing of 100 ml of the vegetative medium. The third seed cultures were incubated at 28 degree and 250 rpm on a rotary shaker for 2 days. Five ml each of the third seed culture was inoculated into 500-ml flask containing 100 ml of the Production Medium B consisting of the following per liter of deionized water: starch, 20 g; yeast extract, 4 g; Hy-Soy, 8

g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The production cultures were incubated at 28 degree C. and 250 rpm on rotary shakers for 1 day. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were added to the production culture. The production culture was further incubated at 28 degree C. and 250 rpm on rotary shaker for 4 days and achieved a titer of 350-400 mg/L for Compound I-16.

[0199] Alternatively, the production of the compounds can be achieved in a 42 L fermentor system using strain NPS21184. Strain NPS21184 was grown in a 500-ml flask containing 100 ml of vegetative medium consisting of the following per liter of deionized water: glucose, 8 g; yeast extract, 6 g; Hy-Soy, 6 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28 degree C. for 3 days on a rotary shaker operating at 250 rpm. Five ml of the first seed culture was inoculated into 500-ml flask containing of 100 ml of the vegetative medium. The second seed cultures were incubated at 28 degree C. and 250 rpm on a rotary shaker for 2 days. Twenty ml each of the second seed culture was inoculated into 2.8 L Fernbach flask containing of 400 ml of the vegetative medium. The third seed cultures were incubated at 28 degree and 250 rpm on a rotary shaker for 2 days. 1.2 L of the third seed culture was inoculated into a 42 L fermentor containing 26 L of Production Medium A. Production Medium B and Production Medium C, with the following composition, can also be used. Production Medium C consisting of the following per liter of deionized water: starch, 15 g; yeast extract 6 g; Hy-Soy, 6 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The fermentor cultures were operated at the following parameters: temperature, 28 degree C.; agitation, 200 rpm; aeration, 13 L/min and back pressure, 4.5 psi. At 36 to 44 hours of the production cycle, approximately 600 grams of sterile Amberlite XAD-7 resin were added to the fermentor culture. The production culture was further incubated at the above operating parameters until day 4 of the production cycle. The aeration rate was lowered to 8 L/min. At day 5 of the production cycle, the fermentor culture achieved a titer of about 300 mg/L for Compound I-16. The culture broth was filtered through cheese cloth to recover the Amberlite XAD-7 resin. The resin was extracted with 2 times 4.5 L liters ethyl acetate followed by 1 time 1.5 liters ethyl acetate. The combined extracts were dried in vacuo. The dried extract was then processed for the recovery of the Compounds of Formulae I-16, I-17, I-20, I-24C, I-26 and I-28.

Example 3

Purification of Compound of Formulae I-16, I-20, I-24C, I-26 and I-28

[0200] The pure compounds of Formulae I-16, I-20 I-24C, I-26 and I-28 were obtained by flash chromatography followed by HPLC. Eight grams crude extract containing 3.8 grams of the compound of Formula I-16 and lesser quantities of I-20, I-24C, I-26 and I-28 was processed by flash chromatography using Biotage Flash40i system and Flash 40M cartridge (K_P-Sil Silica, 32-63 μ m, 90 grams). The flash chromatography was developed by the following step gradient:

[0201] 1. Hexane (1 L)

[0202] 2. 10% Ethyl acetate in hexane (1 L)

- [0203] 3. 20% Ethyl acetate in hexane, first elution (1 L)
 [0204] 4. 20% Ethyl acetate in hexane, second elution (1 L)
 [0205] 5. 20% Ethyl acetate in hexane, third elution (1 L)
 [0206] 6. 25% Ethyl acetate in hexane (1 L)
 [0207] 7. 50% Ethyl acetate in hexane (1 L)
 [0208] 8. Ethyl acetate (1 L)
 [0209] Fractions containing the compound of Formula I-16 in greater or equal to 70% UV purity by HPLC were pooled and subject to HPLC purification, as described below, to obtain I-16, along with I-20 and I-24C, each as pure compounds

Column	Phenomenex Luna 10u Silica
Dimensions	25 cm x 2.1 mm ID
Flow rate	25 μ l/min
Detection	ELSD
Solvent	Gradient of 24% EtOAc/hexane for 19 min, 24% EtOAc/hexane to 100% EtOAc in 1 min, then 100% EtOAc for 4 min

[0210] The fraction enriched in compound of Formula I-16 (described above; ~70% pure with respect to I-16) was dissolved in acetone (60 mg/ml). Aliquots (950 μ l) of this solution were injected onto a normal-phase HPLC column using the conditions described above. Compound I-16 typically eluted after 14 minutes and compounds I-24C and I-26 co-eluted as a single peak at 11 min. When parent samples containing compounds I-17, I-20 and I-28 were processed, compound I-17 eluted at 22 minutes, while I-20 and I-28 co-eluted at 23 minutes during the 100% ethyl acetate wash. Fractions containing compound I-16 and minor analogs were pooled based on composition of compounds present, and evaporated under reduced pressure on a rotary evaporator. This process yielded pure Compound A, as well as separate fractions containing minor compounds I-20, I-24C, I-26 and I-28, which were further purified as described below.

[0211] Sample containing I-24C and I-26 generated from the process described above were further separated using reversed-phase preparative HPLC as follows. The sample containing I-24C (70 mg) was dissolved in acetonitrile at a concentration of 10 mg/ml, and 500 μ l was loaded on an HPLC column of dimensions 21 mm i.d. by 15 cm length containing Eclipse XDB-C18 support. The solvent gradient increased linearly from 15% acetonitrile/85% water to 100% acetonitrile over 23 minutes at a flow rate of 14.5 ml/min. The solvent composition was held at 100% acetonitrile for 3 minutes before returning to the starting solvent mixture. Compound I-26 eluted at 17.5 minutes while compound I-24C eluted at 19 minutes under these conditions.

[0212] Crystalline I-26 was obtained using a vapor diffusion method. Compound I-26 (15 mg) was dissolved in 100 μ l of acetone in a 1.5 ml v-bottom HPLC vial. This vial was then placed inside a larger sealed vessel containing 1 ml of pentane. Crystals suitable for X-ray crystallography experiments were observed along the sides and bottom of the inner vial after 48 hours of incubation at 4° C. Crystallography data was collected on a Bruker SMART APEX CCD X-ray diffractometer (F(000) = 2656, M_{calc} radiation, λ = 0.71073 Å, μ = 0.264 mm⁻¹, T = 100K) at the UCSD Crystallography Lab and the refinement method used was full-matrix least-squares on F². Crystal data N1-2065: C₁₅H₂₀ClNO₄, MW = 313.77, tetragonal, space group P4(1)2(1)₂, a = b = 11.4901(3) Å, c = 46.444(2) Å, α = β = γ = 90°, vol = 6131.6(3) Å³, Z = 16, ρ_{calc} = 1.

360 g cm⁻³, crystal size, 0.30×0.15×0.07 mm³, θ range, 1.75–26.00°, 35367 reflections collected, 6025 independent reflections (R_{int} =0.0480), final R indices ($I>2\sigma(I)$): R_1 =0.0369, wR_2 =0.0794, GOF=1.060.

[0213] In order to separate I-28 from I-20, a reverse-phase isocratic method was employed. Sample (69.2 mg) containing both compounds was dissolved in acetonitrile to a concentration of 10 mg/ml, and 500 μ l was loaded on a reverse-phase HPLC column (ACE 5 C18-HL, 15 cm×21 mm ID) per injection. An isocratic solvent system of 27% acetonitrile/63% water at flow rate of 14.5 ml/min was used to separate compounds I-28 and I-20, which eluted after 14 and 16 minutes, respectively. Fractions containing compounds of interest were immediately evaporated under reduced pressure at room temperature on a rotary evaporator. Samples were then loaded onto a small column of silica and eluted with 10 ml of 70% hexane/30% acetone to remove additional impurities.

[0214] Samples generated from the preparative normal-phase HPLC method described above that contained I-20, but which were free of I-28 could also be triturated with 100% EtOAc to remove minor lipophilic impurities.

[0215] Compound of Formula I-16: UV (Acetonitrile/H₂O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 314 (M+H), 336 (M+Na).

[0216] Compound of Formula I-20: UV (Acetonitrile/H₂O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 266 (M+H); HRMS (ESI), m/z 266.1396 (M+H), Δ_{calc} =1.2 ppm.

[0217] Compound of Formula I-24C: UV (Acetonitrile/H₂O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 328 (M+H), 350 (M+Na); HRMS (ESI), m/z 328.1309 (M+H), Δ_{calc} =2.0 ppm, C₁₆H₂₃NO₄Cl.

[0218] Compound of Formula I-26: UV (Acetonitrile/H₂O) λ_{max} 225(sh) nm; HRMS (ESI), m/z 314.1158 (M+H), Δ_{calc} =0.4 ppm, C₁₅H₂₁NO₄Cl.

[0219] Compound of Formula I-28: UV (Acetonitrile/H₂O) λ_{max} 225(sh) nm; HRMS (ESI), m/z 266.1388 (M+H), Δ_{calc} =1.8 ppm, C₁₄H₂₀NO₄.

Example 4

Fermentation of Compounds of Formulae I-17, I-18, and I-27

[0220] Strain CNB476 was grown in a 500-ml flask containing 100 ml of the first vegetative medium consisting of the following per liter of deionized water: glucose, 4 g; Bacto tryptone, 3 g; Bacto casitone, 5 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28 degree C. for 3 days on a rotary shaker operating at 250 rpm. Five ml of the first seed culture was inoculated into a 500-ml flask containing 100 ml of the second vegetative medium consisting of the following per liter of deionized water: starch, 10 g; yeast extract, 4 g; peptone, 2 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and sodium bromide, 30 g. The second seed cultures were incubated at 28° C. for 7 days on a rotary shaker operating at 250 rpm. Approximately 2 to 3 gram of sterile Amberlite XAD-7 resin were added to the second seed culture. The second seed culture was further incubated at 28° C. for 2 days on a rotary shaker operating at 250 rpm. Five ml of the second seed culture was inoculated into a 500-ml flask containing 100 ml of the second vegetative medium. The third seed culture was incubated at 28° C. for 1 day on a rotary shaker operating at 250 rpm. Approximately 2 to 3 gram of sterile Amberlite XAD-7 resin were added to the

third seed culture. The third seed culture was further incubated at 28° C. for 2 days on a rotary shaker operating at 250 rpm. Five ml of the third culture was inoculated into a 500-ml flask containing 100 ml of the second vegetative medium. The fourth seed culture was incubated at 28° C. for 1 day on a rotary shaker operating at 250 rpm. Approximately 2 to 3 gram of sterile Amberlite XAD-7 resin were added to the fourth seed culture. The fourth seed culture was further incubated at 28° C. for 1 day on a rotary shaker operating at 250 rpm. Five ml each of the fourth seed culture was inoculated into ten 500-ml flasks containing 100 ml of the second vegetative medium. The fifth seed cultures were incubated at 28° C. for 1 day on a rotary shaker operating at 250 rpm. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were added to the fifth seed cultures. The fifth seed cultures were further incubated at 28° C. for 3 days on a rotary shaker operating at 250 rpm. Four ml each of the fifth seed culture was inoculated into one hundred and fifty 500-ml flasks containing 100 ml of the production medium having the same composition as the second vegetative medium. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were also added to the production culture. The production cultures were incubated at 28° C. for 6 day on a rotary shaker operating at 250 rpm. The culture broth was filtered through cheese cloth to recover the Amberlite XAD-7 resin. The resin was extracted with 2 times 3 liters ethyl acetate followed by 1 time 1 liter ethyl acetate. The combined extracts were dried in vacuo. The dried extract, containing 0.42 g of the compound Formula I-17 and 0.16 gram the compound of Formula I-18, was then processed for the recovery of the compounds.

Example 5

Purification of Compounds of Formula I-17, I-18 and I-27

[0221] The pure compounds of Formula I-17 and I-18 were obtained by reversed-phase HPLC as described below:

Column	ACE 5 C18-HL
Dimensions	15 cm × 21 mm ID
Flow rate	14.5 ml/min
Detection	214 nm
Solvent	Gradient of 35% Acetonitrile/65% H ₂ O to 90% Acetonitrile/10% H ₂ O over 15 min

[0222] Crude extract (100 mg) was dissolved in 15 ml of acetonitrile. Aliquots (900 μ l) of this solution were injected onto a reversed-phase HPLC column using the conditions described above. Compounds of Formulae I-17 and I-18 eluted at 7.5 and 9 minutes, respectively. Fractions containing the pure compounds were first concentrated using nitrogen to remove organic solvent. The remaining solution was then frozen and lyophilized to dryness.

[0223] An alternative purification method for Compound I-17 and I-18 was developed for larger scale purification and involved fractionation of the crude extract on a normal phase VLC column. Under these conditions, sufficient amounts of several minor metabolites were identified, including compound I-27. The crude extract (2.4 g) was dissolved in acetone (10 ml) and this solution adsorbed onto silica gel (10 cc) by drying in vacuo. The adsorbed crude extract was loaded on a normal phase silica VLC column (250 cc silica gel, column

dimensions 2.5 cm diameter by 15 cm length) and washed with a step gradient of hexane/EtOAc, increasing in the percentage of hexane in steps of 5% (100 ml solvent per step). The majority of compound I-16 eluted in the 60% hexane/40% EtOAc wash while the majority of compound I-17 eluted in the 50% hexane/50% ethyl acetate wash. Final separation of the compounds was achieved using C18 HPLC chromatography (ACE 5 μ C18-HL, 150 mm \times 21 mm ID) using an isocratic solvent system consisting of 35% ACN/65% H₂O. Under these conditions, compound I-27 eluted at 11 minutes, compound I-17 eluted at 12.00 minutes, traces of compound A eluted at 23.5 minutes, and compound I-18 eluted at 25.5 minutes. The resulting samples were dried in vacuo using no heat to remove the aqueous solvent mixture. The spectroscopic data for these samples of compound I-16 and compound I-18 were found to be identical with those of samples prepared from earlier purification methods. The sample of compound I-18 was found to contain 8% of the lactone hydrolysis product and was further purified by washing through a normal phase silica plug (1 cm diameter by 2 cm height) and eluting using a solvent mixture of 20% EtOAc/80% Hexanes (25 ml). The resulting sample was found to contain pure compound I-18.

[0224] The fractions containing compound I-27 described above were further purified using normal phase semipreparative HPLC (Phenomenex Luna Si 10 μ , 100 Å; 250 \times 10 mm id) using a solvent gradient increasing from 100% hexane to 100% EtOAc over 20 minutes with a flowrate of 4 ml/min. Compound I-27 eluted as a pure compound after 11.5 minutes (0.8 mg, 0.03% isolated yield from dried extract weight).

[0225] Compound of Formula I-17: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm. High Res. Mass (APCI): m/z 280.156 (M+H), Δ_{calc} = 2.2 ppm, C₁₅H₂₂NO₄.

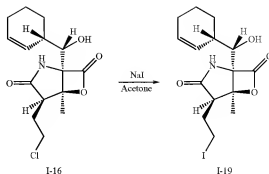
[0226] Compound of Formula I-18: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm. High Res. Mass (APCI): m/z 358.065 (M+H), Δ_{calc} = 1.9 ppm, C₁₅H₂₁NO₄Br.

[0227] Compound I-27: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm; MS (HR-ESI), m/z 280.1556 (M+H) Δ_{calc} = 2.7 ppm (C₁₅H₂₂NO₄); ¹H NMR (DMSO-d₆).

Example 6

Preparation of Compound of Formula I-19 from I-16

[0228] A sample of compound of Formula I-16 (250 mg) was added to an acetone solution of sodium iodide (1.5 g in 10 ml) and the resulting mixture stirred for 6 days. The solution was then filtered through a 0.45 micron syringe filter and injected directly on a normal phase silica HPLC column (Phenomenex Luna 10 μ Silica, 25 cm \times 21.2 mm) in 0.95 ml aliquots. The HPLC conditions for the separation of compound formula I-19 from unreacted I-16 employed an isocratic HPLC method consisting of 24% ethyl acetate and 76% hexane, in which the majority of compound I-19 eluted 2.5 minutes before compound I-16. Equivalent fractions from each of 10 injections were pooled to yield 35 mg compound I-19. Compound I-19: UV (Acetonitrile/H₂O) 225 (sh), 255 (sh) nm; ESMs, m/z 406.0 (M+H); HRMS (ESI), m/z 406.0513 [M+H]⁺, Δ_{calc} = -0.5 ppm, C₁₅H₂₁NO₄I; ¹H NMR in DMSO-d₆.



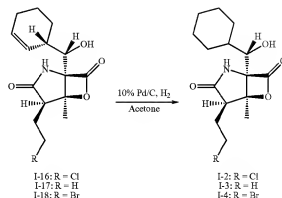
Example 7

Synthesis of the Compounds of Formulae I-2, I-3, and I-4

[0229] Compounds of Formulae I-2, I-3 and I-4 can be synthesized from compounds of Formulae I-16, I-17 and I-18, respectively, by catalytic hydrogenation.

Exemplary Depiction of Synthesis

[0230]



Example 7A

Catalytic Hydrogenation of Compound of Formula I-16

[0231] Compound of Formula I-16 (10 mg) was dissolved in acetone (5 mL) in a scintillation vial (20 mL) to which was added the 10% (w/w) Pd/C (1-2 mg) and a magnetic stirrer bar. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 3 cc silica column and washed with acetone. The filtrate was filtered again through 0.2 μ m Gelman Acrodise to remove any traces of catalyst. The solvent was evaporated off from filtrate under reduced pressure to yield the compound of Formula I-2 as a pure white powder: UV (acetonitrile/H₂O): λ_{max} 225 (sh) nm.

Example 7B

Catalytic Hydrogenation of Compound of Formula I-17

[0232] Compound of Formula I-17 (5 mg) was dissolved in acetone (3 mL) in a scintillation vial (20 mL) to which was

added the 10% (w/w) Pd/C (about 1 mg) and a magnetic stirrer bar. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodisc to remove the catalyst. The solvent was evaporated off from filtrate to yield the compound of Formula I-3 as a white powder which was purified by normal phase HPLC using the following conditions:

Column:	Phenomenex Luna 10 μ Silica
Dimensions:	25 cm \times 21.2 mm ID
Flow rate:	14.5 ml/min
Detection:	ELSD
Solvent:	5% to 60% EtOAc/Hex for 19 min, 60 to 100% EtOAc in 1 min, then 4 min at 100% EtOAc

[0233] Compound of Formula I-3 eluted at 22.5 min as a pure compound: UV (acetonitrile/H₂O): λ_{max} 225 (sh) nm. Formula I-3: m/z 282 (M+H), 304 (M+Na).

Example 7C

Catalytic Hydrogenation of Compound of Formula I-18

[0234] 3.2 mg of compound of Formula I-18 was dissolved in acetone (3 mL) in a scintillation vial (20 mL) to which was added the 10% (w/w) Pd/C (about 1 mg) and a magnetic stirrer bar. The reaction mixture was stirred in hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodisc to remove the catalyst. The solvent was evaporated off from filtrate to yield the compound of Formula I-4 as a white powder which was further purified by normal phase HPLC using the following conditions:

Column:	Phenomenex Luna 10 μ Silica
Dimensions:	25 cm \times 21.2 mm ID
Flow rate:	14.5 ml/min
Detection:	ELSD
Solvent:	5% to 80% EtOAc/Hex for 19 min, 80 to 100% EtOAc in 1 min, then 4 min at 100% EtOAc

[0235] Compound of Formula I-4 eluted at 16.5 min as a pure compound: UV (acetonitrile/H₂O): λ_{max} 225 (sh) nm. Formula I-4: m/z 360 (M+H), 382 (M+Na).

[0236] In addition, high resolution mass spectrometry data were obtained for compounds I-2, I-3, and I-4. Compound I-2: HRMS (ESI), m/z 316.1305 [M+H]⁺, Δ_{calc} = -3.5 ppm, C₁₅H₂₃NO₄Cl. Compound I-3: HRMS (ESI), m/z 282.1706 [M+H]⁺, Δ_{calc} = 0.3 ppm, C₁₃H₂₃NO₄. Compound I-4: HRMS (ESI), m/z 360.0798 [M+H]⁺, Δ_{calc} = -3.4 ppm, C₁₅H₂₃NO₄Br.

Example 8

Synthesis of the Compounds of Formulae I-5A and I-5B

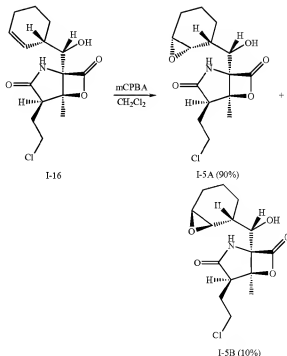
[0237] Compounds of Formula I-5A and Formula I-5B can be synthesized from compound of Formula I-16 by epoxidation with mCPBA.

[0238] Compound of Formula I-16 (101 mg, 0.32 mmole) was dissolved in methylenechloride (30 mL) in a 100 ml of round bottom flask to which was added 79 mg (0.46 mmole) of meta-chloroperoxybenzoic acid (mCPBA) and a magnetic stirrer bar. The reaction mixture was stirred at room temperature for about 18 hours. The reaction mixture was poured onto a 20 cc silica flash column and eluted with 120 mL of CH₂Cl₂, 75 mL of 1:1 ethyl acetate/hexane and finally with 40 mL of 100% ethyl acetate. The 1:1 ethyl acetate/hexane fractions yield a mixture of diastereomers of epoxyderivatives, Formula I-5A and I-5B, which were separated by normal phase HPLC using the following conditions:

Column	Phenomenex Luna 10 μ Silica
Dimensions	25 cm \times 21.2 mm ID
Flow rate	14.5 ml/min
Detection	ELSD
Solvent	25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc in 1 min, then 5 min at 100% EtOAc

[0239] Compound Formula I-5A (major product) and I-5B (minor product) eluted at 21.5 and 19 min, respectively, as pure compounds. Compound I-5B was further chromatographed on a 3 cc silica flash column to remove traces of chlorobenzoic acid reagent.

Chemical Structures:



Structural Characterization

[0240] Formula 1-5A: UV (Acetonitrile/ H_2O) λ_{max} 225 (sh) nm. Low Res. Mass: m/z 330 (M+H), 352 (M+Na); HRMS (ESI), m/z 330.1099 [M+H]⁺, $\Delta_{calc} = -2.9$ ppm, $C_{15}H_{21}NO_3Cl$.

[0241] Formula 1-5B: UV (Acetonitrile/ H_2O) λ_{max} 225 (sh) nm. Low Res. Mass: m/z 330 (M+H), 352 (M+Na); HRMS (ESI), m/z 330.1105 [M+H]⁺, $\Delta_{calc} = -0.9$ ppm, $C_{15}H_{21}NO_3Cl$.

Example 9

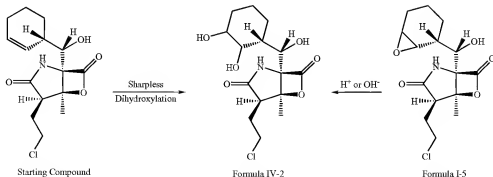
Synthesis of the Compounds of Formulae IV-1, IV-2, IV-3 and IV-4

Synthesis of Diol Derivatives (Formula IV-2)

[0242] Diols can be synthesized by Sharpless dihydroxylation using AD mix- α and β : AD mix- α is a premix of four reagents, $K_2OsO_2(OH)_4$, K_2CO_3 , $K_3Fe(CN)_6$, (DHQD)₂-PHAL [1,4-bis(9-O-dihydroquinine)phthalazine] and AD mix- β is a premix of $K_2OsO_2(OH)_4$, K_2CO_3 , $K_3Fe(CN)_6$, (DHQD)₂-PHAL [1,4-bis(9-O-dihydroquinidine)phthalazine] which are commercially available from Aldrich. Diol can also be synthesized by acid or base hydrolysis of epoxy compounds (Formula 1-5A and 1-5B) which may be different to that of products obtained in Sharpless dihydroxylation in their stereochemistry at carbons bearing hydroxyl groups

Sharpless Dihydroxylation of Compounds 1-16, 1-17 and 1-18

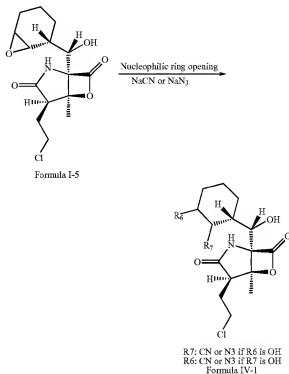
[0243] Any of the compounds of Formulae 1-16, 1-17 and 1-18 can be used as the starting compound. In the example below, compound of Formula 1-16 is used. The starting compound is dissolved in *t*-butanol/water in a round bottom flask to which is added AD mix- α or β and a magnetic stir bar. The reaction is monitored by silica TLC as well as mass spectrometer. The pure diols are obtained by usual workup and purification by flash chromatography or HPLC. The structures are confirmed by NMR spectroscopy and mass spectrometry. In this method both hydroxyl groups are on same side.



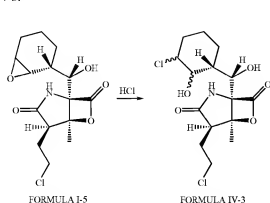
Nucleophilic Ring Opening of Epoxy Compounds (1-5):

[0244] The epoxy ring is opened with various nucleophiles like NaCN, NaN₃, NaOAc, HBr, HCl, etc. to create various substituents on the cyclohexane ring, including a hydroxyl substituent.

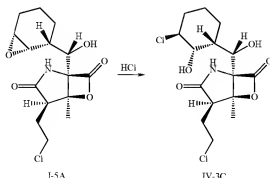
Examples

[0245]

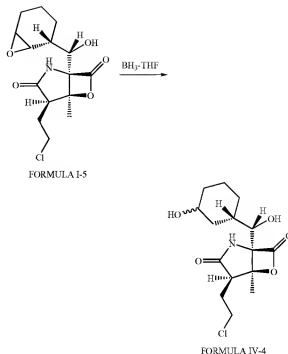
[0246] The epoxy is opened with HCl to make Formula IV-3:



[0247] Compound of Formula I-5A (3.3 mg) was dissolved in acetonitrile (0.5 ml) in a 1 dram vial to which was added 5% HCl (500 μ l) and a magnetic stir bar. The reaction mixture was stirred at room temperature for about an hour. The reaction was monitored by mass spectrometry. The reaction mixture was directly injected on normal phase HPLC to obtain compound of Formula IV-3C as a pure compound without any work up. The HPLC conditions used for the purification were as follows: Phenomenex Luna 10 μ Silica column (25 cm \times 21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc in 1 min, then 5 min at 100% EtOAc at a flow rate of 14.5 ml/min. An ELSD was used to monitor the purification process. Compound of Formula IV-3C eluted at about 18 min (2.2 mg). Compound of Formula IV-3C: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm; ES/MS, m/z 366 (M+H)⁺, 388 (M+Na)⁺; HRMS (ESI), m/z 366.0875 [M+H]⁺, Δ_{calc} =0.0 ppm, C₁₅H₂₂NO₄Cl₂; ¹H NMR in DMSO-*d*₆, the stereochemistry of the compound of Formula IV-3C was determined based on coupling constants observed in the cyclohexane ring in 1:1 C₆D₆/DMSO-*d*₆.



[0248] Reductive ring opening of epoxides (I-5): The compound of Formula I-5 is treated with metalhydrides like BH₃-THF complex to make compound of Formula IV-4.



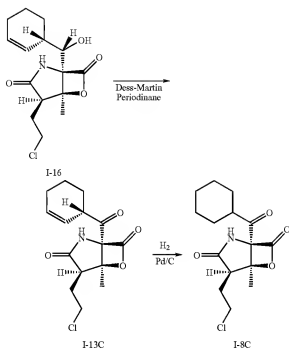
Example 10

Synthesis of the Compounds of Formulae I-13C and I-8C

[0249] Compound of Formula I-16 (30 mg) was dissolved in CH₂Cl₂ (6 ml) in a scintillation vial (20 ml) to which Dess-Martin Periodinane (122 mg) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 2 hours. The progress of the reaction was monitored by TLC (Hex:EtOAc, 6:4) and analytical HPLC. From the reaction mixture, the solvent volume was reduced to one third, absorbed on silica gel, poured on top of a 20 cc silica flash column and eluted in 20 ml fractions using a gradient of Hexane/EtOAc from 10 to 100%. The fraction eluted with 30% EtOAc in Hexane contained a mixture of rotamers of Formula I-13C in a ratio of 1.5:8.5. The mixture was further purified by normal phase HPLC using the Phenomenex Luna 10 μ Silica column (25 cm \times 21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc over 1 min, holding at 100% EtOAc for 5 min, at a flow rate of 14.5 ml/min. An ELSD was used to monitor the purification process. Compound of Formula I-13C eluted at 13.0 and 13.2 mins as a mixture of rotamers with in a ratio of 1.5:8.5 (7 mg). Formula I-13C: UV (Acetonitrile/H₂O) λ_{max} 226 (sh) & 300 (sh) nm; ES/MS, m/z 312 (M+H)⁺, 334 (M+Na)⁺; HRMS (ESI), m/z 312.1017 [M+H]⁺, Δ_{calc} =-4.5 ppm, C₁₅H₁₉NO₄Cl; ¹H NMR in DMSO-*d*₆.

[0250] The rotamer mixture of Formula I-13C (4 mg) was dissolved in acetone (1 ml) in a scintillation vial (20 ml) to which a catalytic amount (0.5 mg) of 10% (w/w) Pd/C and a magnetic stir bar were added. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodisc to remove the catalyst. The solvent was evaporated from the filtrate to yield compound of Formula I-8C as a colorless gum which was further purified by

normal phase HPLC using a Phenomenex Luna 10u Silica column (25 cm×21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc over 1 min, holding at 100% EtOAc for 5 min, at a flow rate of 14.5 ml/min. An ELSD was used to monitor the purification process. Compound of Formula I-8C (1 mg) eluted at 13.5 min as a pure compound. Formula I-8C: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm; ESMS, *m/z* 314 (M+H)⁺, 336 (M+Na)⁺; HRMS (ESI), *m/z* 314.1149 [M+H]⁺, Δ_{calc} -3.3 ppm, C₁₅H₂₁NO₄Cl; ¹H NMR in DMSO-d₆.

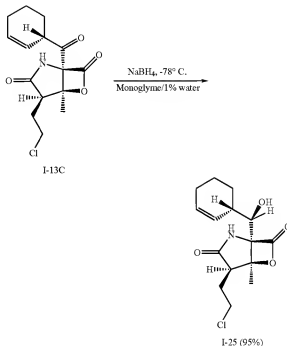


Example 11

Synthesis of the Compound of Formula I-25 from I-13C

[0251] The rotamer mixture of Formula I-13C (5 mg) was dissolved in dimethoxy ethane (monoglyme; 1.5 ml) in a scintillation vial (20 ml) to which water (15 μ l (1% of the final solution concentration)) and a magnetic stir bar were added. The above solution was cooled to -78° C. on a dry ice-acetone bath, and a sodium borohydride solution (3.7 mg of NaBH₄ in 0.5 ml of monoglyme (created to allow for slow addition)) was added drop-wise. The reaction mixture was stirred at -78° C. for about 14 minutes. The reaction mixture was acidified using 2 ml of 4% HCl solution in water and extracted with CH₂Cl₂. The organic layer was evaporated to yield mixture of compound of formulae I-25 and I-16 in a 9.5:0.5 ratio as a white solid, which was further purified by normal phase HPLC using a Phenomenex Luna 10u Silica column (25 cm×21.2 mm ID). The mobile phase was 24% EtOAc/76% Hexane, which was held isocratic for 19 min, followed by a linear gradient of 24% to 100% EtOAc over 1 min, and held at 100% EtOAc for 5 min; the flow rate was 25 ml/min. An ELSD was used to monitor the purification process. Compound of formula I-25 (1.5 mg) eluted at 11.64 min as a pure

compound. Compound of Formula I-25: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm; ESMS, *m/z* 314 (M+H)⁺, 336 (M+Na)⁺; HRMS (ESI), *m/z* 314.1154 [M+H]⁺, Δ_{calc} -0.6 ppm, C₁₅H₂₁NO₄Cl; ¹H NMR in DMSO-d₆.



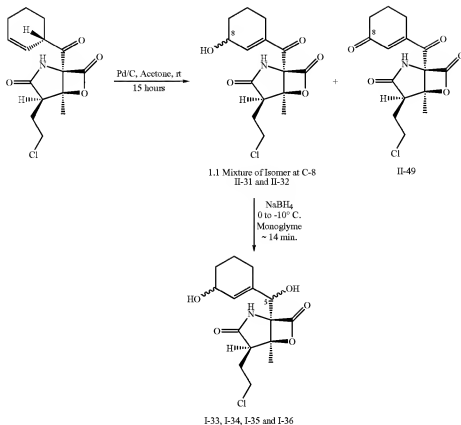
Example 12

Synthesis of the Compounds of Formulae I-31, I-32 and I-49 from I-13C; and Compounds of Formulae I-33, I-34, I-35 and I-36 from I-31 and I-32

[0252] A rotamer mixture of the Compound of Formula I-13C (20 mg) was dissolved in acetone (4 ml) in a scintillation vial (20 ml) to which a catalytic amount (3 mg) of 10% (w/w) Pd/C and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodise to remove the catalyst. The solvent was evaporated from the filtrate to yield a mixture of diastereomers of hydroxy derivatives of Formulae I-31 and I-32 (1:1) and a minor compound I-49, which were separated by reversed phase HPLC using Ace 5u C18 column (150 mm×22 mm ID) with a solvent gradient of 90% to 30% H₂O/Acetonitrile over 15 min, 70 to 100% Acetonitrile over 5 min, holding at 100% Acetonitrile for 4 min, at a flow rate of 14.5 ml/min. A diode array detector was used to monitor the purification process. Compound I-31 (2 mg), I-32 (2 mg) and I-49 (0.2 mg) eluted at 10.6, 10.8 and 11.54 min, respectively, as pure compounds. I-31: UV (Acetonitrile/H₂O) λ_{max} 250 (sh) nm; ESMS *m/z* 328.1 (M+H)⁺ & 350.0 (M+Na)⁺. I-32: UV (Acetonitrile/H₂O) λ_{max} 250 (sh) nm; ESMS, *m/z* 328.1 (M+H)⁺ & 350.0 (M+Na)⁺. I-49: UV (Acetonitrile/H₂O) λ_{max} 250 (sh) and 320 nm; ESMS, *m/z* 326.0 (M+H)⁺, 343.1 (M+H₂O)⁺ & 348.0 (M+Na)⁺.

[0253] In an alternate method, compounds I-31, I-32 and I-49 were separated by normal phase HPLC using Phenomenex Luna 10u Silica column (25 cm×21.2 mm ID) with a solvent gradient of 10% to 100% Hexane/EtOAc over 24 min, holding at 100% EtOAc for 5 min, at a flow rate of 14.5 ml/min. ELSD was used to monitor the purification process.

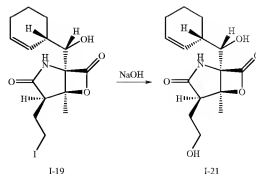
[0254] The ketone of the compounds of formula 1-31 and 1-32 can be reduced by using sodium borohydride at 0 to -10° C. in monoglyme solvent for about 14 minutes. The reaction mixture can be acidified using 4% HCl solution in water and extracted with CH₂Cl₂. The organic layer can be evaporated to yield the mixtures of compounds of formulae 1-33, 1-34, 1-35 and 1-36 which can be separated by chromatographic methods.



Example 13

Synthesis of the Compound of Formulae 1-21 from 1-19

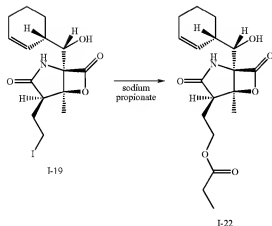
[0255] Acetone (7.5 ml) was vigorously mixed with 5 N NaOH (3 ml) and the resulting mixture evaporated to a minimum volume in vacuo. A sample of 100 µl of this solution was mixed with compound of Formula 1-19 (6.2 mg) in acetone (1 ml) and the resulting biphasic mixture vortexed for 2 minutes. The reaction solution was immediately subjected to preparative C18 HPLC. Conditions for the purification involved a linear gradient of 10% acetonitrile/90% water to 90% acetonitrile/10% water over 17 minutes using an Ace S₁₈ C18 HPLC column of dimensions 22 mm id by 150 mm length. Compound of Formula 1-21 eluted at 9.1 minutes under these conditions to yield 0.55 mg compound. Compound of Formula 1-21: UV (Acetonitrile/H₂O) 225 (sh), ESMS, m/z 296.1 (M+H)⁺; ¹H NMR in DMSO-d₆.



Example 14

Synthesis of the Compound of Formulae 1-22 from 1-19

[0256] A sample of 60 mg sodium propionate was added to a solution of compound of Formula 1-19 (5.3 mg) in DMSO (1 ml) and the mixture sonicated for 5 minutes, though the sodium propionate did not completely dissolve. After 45 minutes, the solution was filtered through a 0.45 μ syringe filter and purified directly using HPLC. Conditions for the purification involved a linear gradient of 10% acetonitrile/90% water to 90% acetonitrile/10% water over 17 minutes using an Ace 5 μ C18 HPLC column of dimensions 22 mm id by 150 mm length. Under these conditions, compound of Formula 1-22 eluted at 12.3 minutes to yield 0.7 mg compound (15% isolated yield). UV (Acetonitrile/H₂O) 225 (sh), ESMS, *m/z* 352.2 (M+H)⁺; HRMS (ESI), *m/z* 352.1762 [M+H]⁺, $\Delta_{\text{calc}} = -0.6$ ppm, C₁₈H₂₆NO₆; ¹H NMR in DMSO-*d*₆.



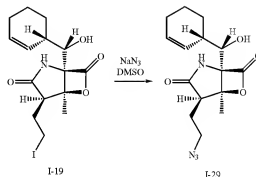
Example 15

Synthesis of the Compound of Formula 1-29 from 1-19

[0257] A sample of NaN₃ (80 mg) was dissolved in DMSO (1 ml) and transferred to a vial containing Compound 1-19 (6.2 mg) which was contaminated with approximately 10% Compound 1-16. The solution was incubated at room temperature for 1 hr prior to purification on C18 HPLC (ACE 5 μ C18-HL, 150 mmx21 mm ID) using a solvent gradient of 10% acetonitrile/90% H₂O to 90% acetonitrile/10% H₂O over 17 minutes. Using this method, the desired azido derivative 1-29 co-eluted with Compound 1-16 contaminant at 12.5

minutes (4.2 mg, 85% yield). A 2.4 mg portion of compound 1-29 was further purified using additional C18 HPLC chromatography (ACE 5 μ C18-HL, 150 mmx21 mm ID) using an isocratic solvent gradient consisting of 35% acetonitrile/65% H₂O. Under these conditions compound 1-29 eluted after 20 minutes, while Compound 1-16 eluted after 21.5 minutes. The resulting sample consisted of 1.1 mg Compound 1-29 was used for characterization in biological assays.

[0258] Compound 1-29: UV (Acetonitrile/H₂O) 225 (sh), ESMS, *m/z* 321.1 (M+H)⁺; ¹H NMR in DMSO-*d*₆.



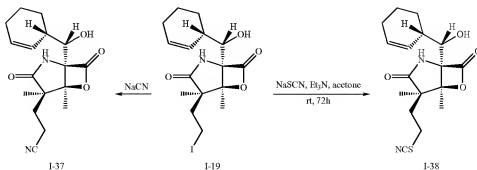
Example 16

Synthesis of the Compounds of Formulae 1-37 and 1-38 from 1-19

[0259] The compounds of Formulae 1-37 and 1-38 can be prepared from the compound of Formula 1-19 by cyano-dehalogenation or thiocyanato-dehalogenation, respectively. Compound 1-19 can be treated with NaCN or KCN to obtain compound 1-37. Alternatively, Compound 1-19 can be treated with NaSCN or KSCN to obtain compound 1-38.

Synthesis of the compound of Formula 1-38 from 1-19:

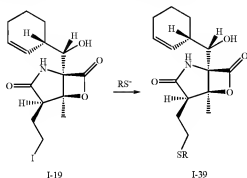
[0260] The compound of formula 1-19 (10.6 mg, 0.02616 mmol) was dissolved in 1.5 ml of acetone in a scintillation vial (20 ml) to which sodium thiocyanate (10.0 mg, 0.1234 mmol), triethylamine (5 μ l, 0.03597 mmol) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for 72 hours. The reaction mixture was concentrated in vacuo to yield the compound 1-38. Compound 1-38 was purified by normal phase HPLC using a Phenomenex Luna 10 μ Silica column (25 cmx21.2 mm ID) with a solvent gradient of 0 to 95% H₂O/Acetonitrile over 21 min, at a flow rate of 14.5 ml/min. Diode array detector was used to monitor the purification process. Compound 1-38 (3.0 mg, 34% yield) eluted at 18.0 min as a pure compound. 1-38: UV Acetonitrile/H₂O λ_{max} 203 (sh) nm; ESMS *m/z* 337.1 (M+H)⁺ & 359.1 (M+Na)⁺.



Example 17

Synthesis of the Compound of Formula I-39 from I-19

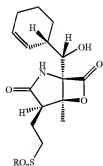
[0261] Thiols and thioethers of the Formula I-39 can be formed by dehalogenation of the compound of Formula I-19. Thiols ($R=H$) can be formed by treatment of Compound I-19 with $NaSH$, for example, while thioethers ($R=alkyl$) can be formed by treatment of Compound I-19 with salts of thiols, or alternatively, by treatment with thiols themselves by running the reaction in benzene in the presence of DIBU.



Example 18

Synthesis of the Compound of Formula I-40 from I-39

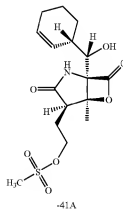
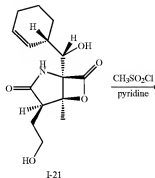
[0262] Sulfoxides ($n=1$) and sulfones ($n=2$) of the Formula I-40 can be formed by oxidation of thioethers of the Formula I-39, for example, with hydrogen peroxide or other oxidizing agents.



Example 19

Synthesis of the Compound of Formula I-41A from I-21

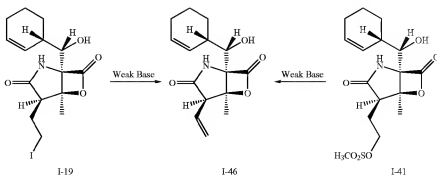
[0263] The compound of the Formula I-41A can be prepared by treatment of the compound of Formula I-21 (or a protected derivative of I-21, where the C-5 alcohol or lactam NH are protected, for example) with methyl sulfonyl chloride (mesyl chloride) in pyridine, for example, or by treatment with mesyl chloride in the presence of triethylamine. Other sulfonate esters can be similarly prepared.



Example 20

Synthesis of the Compound of Formula I-46 from I-19 OR I-41A

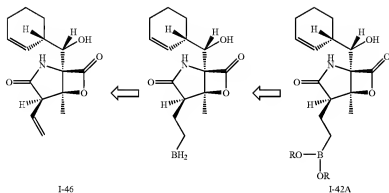
[0264] The alkene of the Formula I-46 can be prepared by dehydroiodination of the compound of Formula I-19, or by hydro-mesyloxy elimination of the compound of Formula I-41A, for example, by treatment with base.



Example 21

Synthesis of the Compound of Formula I-42A

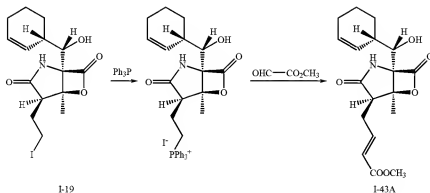
[0265] Synthesis of boronic acids or esters, for example, the compound of the Formula I-42A, can be achieved as outlined in the retrosynthetic scheme below. Hydroboration of the alkene of Formula I-46 gives the corresponding alkyl borane, which can be converted to the corresponding boronic acid or ester, for example, the compound of the Formula I-42A.



Example 22

Synthesis of the Compound of Formula I-43A

[0266] The compound of the Formula I-43A can be prepared by treatment of the compound of Formula I-19 with triphenyl phosphine to make a phosphorus ylide, which can be treated with various aldehydes, for example, glyoxylic acid methyl ester, to make Formula I-43A.



Example 23

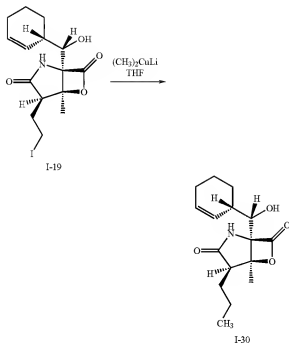
Synthesis of the Compound of Formula I-30 from I-19

[0267] A portion of CuI (100 mg) was placed in a 25 ml pear bottom flask and flushed with Ar gas for 30 minutes. Ar gas flow was maintained through the flask throughout the course of the reaction. The vessel was cooled to -78°C . prior to addition of dry THF (5 ml) followed by the immediate dropwise addition of a solution of methylolithium in dry THF

(5.0 ml, 1.6 M) with vigorous stirring. A solution of Compound I-19 in dry THF (12 mg Compound I-19, 1 ml THF) was added slowly to the clear dialkylcuprate solution and the resulting mixture stirred at -78°C . for 1 hr. The reaction was quenched by washing the THF solution through a plug of silica gel (1 cm diameter by 2 cm length) along with further washing using a solution of 50% EtOAc/50% hexanes (50 ml). The combined silica plug washes were dried in vacuo and subjected to further C18 HPLC purification in 2 injections (ACE 5 μ C18-HL, 150 mmx21 mm ID) using an isocratic

solvent gradient consisting of 35% acetonitrile/65% H₂O. Compound I-30 eluted under these conditions at 23.5 minutes and yielded 2.4 mg material (27% isolated yield) at 90.8% purity as measured by analytical HPLC. An alternative normal phase purification method can be utilized using Phenomenex Luna 10 μ Silica column (25 cm \times 21.2 mm ID) with a solvent gradient consisting of 100% hexanes/ethyl acetate to 0% hexanes over 20 minutes. Compound I-30 eluted under these conditions at 16.5 minutes and yielded 3.0 mg material (41% isolated yield) at 97.1% purity as measured by analytical HPLC.

[0268] Compound I-30: UV (Acetonitrile/H₂O) 225 (sh), ESMS, *m/z* 294.1 (M+H)⁺; HRMS (ESI), *m/z* 294.1696 [M+H]⁺, Δ_{calc} -3.2 ppm, C₁₆H₁₂N₂O₄; ¹H NMR in DMSO-*d*₆.



[0269] Compound I-30 can also be obtained by saline fermentation of strain CNB476. In one example, CNB476 was transferred to 500-mL flasks containing 100 mL production medium consisting of the following per liter of deionized water: starch, 10 g; yeast extract, 4 g; Hy-Soy, 4 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and synthetic sea salt, 30 g. The production cultures were incubated at 28° C. and 250 rpm for 1 day. Approximately 2 g of sterile Amberlite XAD-7 resin was added to the production cultures. The production cultures were further incubated for 5 days. The resin was recovered from the broth and extracted with ethyl acetate. The extract was dried in vacuo. The dried extract (8 g) was then processed for the recovery of Compound I-30.

[0270] The crude extract was processed by flash chromatography using a Biotage Flash system. The flash chromatography was developed by the following step gradient: i) Hexanes (1 L); 1) 10% EtOAc in hexanes (1 L); ii) 20% EtOAc in hexanes, first elution (1 L); iv) 20% EtOAc in hexanes, second elution (1 L); v) 20% EtOAc in hexanes, third elution (1

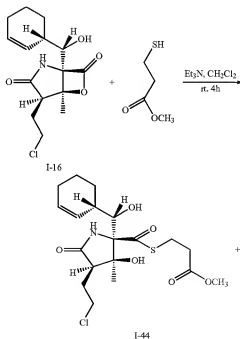
L); vi) 25% EtOAc in hexanes (1 L); vii) 50% EtOAc in hexanes (1 L); viii) EtOAc (1 L). Fractions containing Compound I-30 was further purified by normal phase HPLC using an isocratic solvent system of 24% EtOAc/hexanes followed by a 100% EtOAc. Compound I-30 eluted 22 minutes into the isocratic portion of the run.

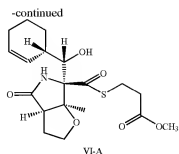
[0271] Fractions enriched in Compound I-30 were further processed by normal phase HPLC using a 27 minute linear gradient from 15% hexanes/85% EtOAc to 100% EtOAc. Compound I-30 eluted after 15 min.

Example 24

Synthesis of the Compound of Formulae I-44 and VI-1A from I-16

[0272] The compound of Formula I-16 (30 mg, 0.096 mmol) was dissolved in CH₂Cl₂ (9 ml) in a scintillation vial (20 ml) to which triethylamine (40 μ L, 0.29 mmol), methyl-3-mercapto propionate (thiol, 250 μ L) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 4 hours. The solvent was evaporated from the reaction mixture to yield a mixture of compounds of Formulae I-44 and VI-1A (19:1), which were separated by reversed phase HPLC using Ace 5u C18 column (150 mm \times 22 mm ID) with a solvent gradient of 35% to 90% H₂O/Acetonitrile over 17 min, 90 to 100% Acetonitrile over 1 min, holding at 100% Acetonitrile for 1 min, at a flow rate of 14.5 mL/min. Diode array detector was used to monitor the purification process. Compounds I-44 (20 mg) and VI-1A (1 mg) eluted at 11.68 and 10.88 min, respectively, as pure compounds. Compound I-44: UV (Acetonitrile/H₂O) λ_{max} 240 (sh) nm; ESMS *m/z* 434.0 (M+H)⁺ & 456.0 (M+Na)⁺. Compound VI-1A: UV (Acetonitrile/H₂O) λ_{max} 220 (sh) nm; ESMS, *m/z* 398.0 (M+H)⁺ & 420.0 (M+Na)⁺.





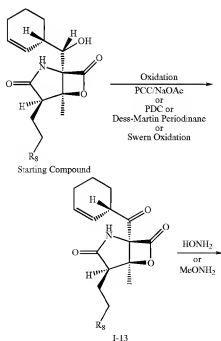
Example 25

Oxidation of Secondary Hydroxyl Group in Compounds of Formulae I-16, I-17 and I-18 and Reaction with Hydroxy or Methoxy Amines

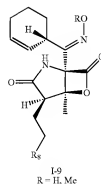
[0273] Any of the compounds of Formulae I-16, I-17 and I-18 can be used as the starting compound. The secondary hydroxyl group in the starting compound is oxidized using either of the following reagents: pyridinium dichromate (PDC), pyridinium chlorochromate (PCC), Dess-Martin periodinane or oxalyl chloride (Swern oxidation) (Ref: Organic Syntheses, collective volumes I-VII). Preferably, Dess-Martin periodinane can be used as a reagent for this reaction. (Ref: Fenteany G. et al. Science, 1995, 268, 726-73). The resulting keto compound is treated with hydroxylamine or methoxy amine to generate oximes.

Examples

[0274]



-continued



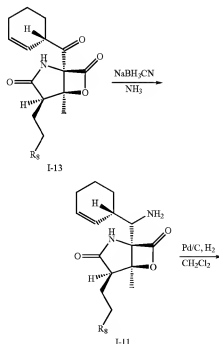
Example 26

Reductive Amination of Keto-Derivative

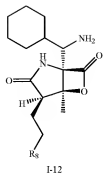
[0275] The keto derivatives, for example Formula I-8 and I-13, are treated with sodium cyanoborohydride (NaBH₃CN) in the presence of various bases to yield amine derivatives of the starting compounds which are subsequently hydrogenated with 10% Pd/C, H₂ to reduce the double bond in the cyclohexene ring.

Example

[0276]

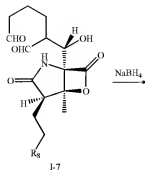


-continued



I-12

-continued



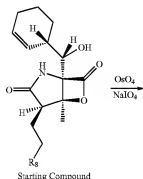
I-7

Example 27**Cyclohexene Ring Opening**

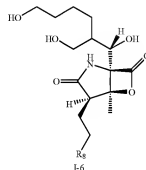
[0277] Any compound of Formulae I-16, I-17 and I-18 can be used as a starting compound. The Starting Compounds can be protected, for example, at the alcohol and/or at the lactam nitrogen positions, and treated with OsO_4 and NaIO_4 in $\text{THF}-\text{H}_2\text{O}$ solution to yield dial derivatives which are reduced to the alcohol with NaBH_4 . The protecting groups can be removed at the appropriate stage of the reaction sequence to produce I-7 or I-6.

Example

[0278]



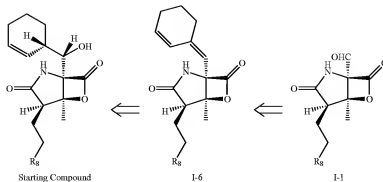
Starting Compound



I-6

Example 28**Dehydration of Alcohol Followed by Aldehyde Formation at Lactone-Lactam Ring Junction**

[0279] A starting compound of any of Formulae I-16, I-17 or I-18 is dehydrated, for example, by treatment with mesylchloride in the presence of base, or, for example, by treatment with Burgess reagent or other dehydrating agents. The resulting dehydrated compound is treated with OsO_4 , followed by NaIO_4 , or alternatively by ozonolysis, to yield an aldehyde group at the lactone-lactam ring junction.



Starting Compound

I-6

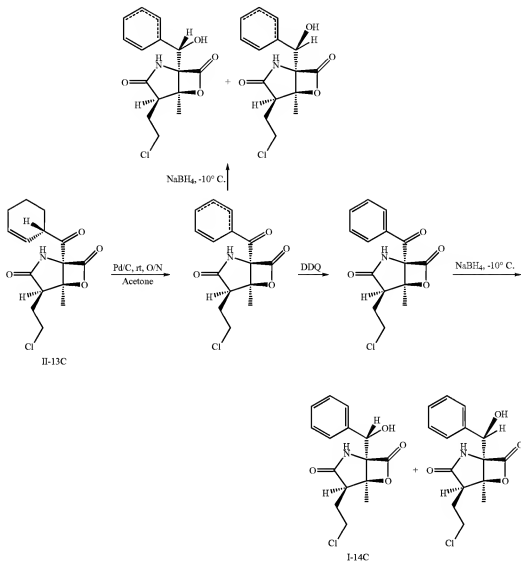
I-1

Example 29

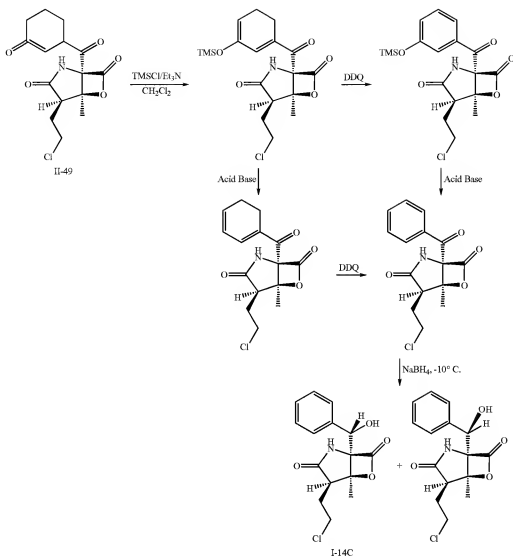
Oxidation of the Cyclohexene Ring to Produce
Cyclohexadienes or a Phenyl Ring

[0280] A Starting Compound, such as the ketone of Formula I-13C, is treated with Pd/C to produce a cyclohexadiene derivative. The new double bond can be at any position of the

cyclohexene ring. The ketone can be reduced, for example, with sodium borohydride, to obtain the corresponding secondary alcohol(s). Alternatively, the cyclohexadiene derivative can be further treated, for example with DDQ, to aromatize the ring to a phenyl group. Similarly, the ketone can be reduced, for example, with sodium borohydride, to obtain the corresponding secondary alcohol(s).



[0281] As an alternate method, the starting compound, such as the compound of Formula I-49, can be treated, for example with TMSCl to produce cyclohexadiene derivative. The cyclohexadiene derivative can be further treated, for example with DDQ , to aromatize the ring to a phenyl group. The OTMS on the phenyl group can be removed, for example, with acid or base. Similarly, the ketone can be reduced, for example, with sodium borohydride, to obtain the corresponding secondary alcohol(s).

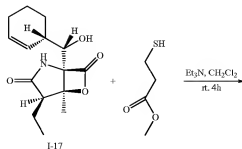


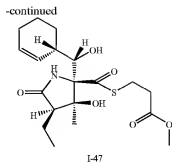
Example 30

Synthesis of the Compound of Formula I-47 from I-17

[0282] The compound of Formula I-17 (25 mg, 0.0896 mmol) was dissolved in CH₂Cl₂ (9 ml) in a scintillation vial (20 ml) to which triethylamine (38 μ l, 0.27 mmol), methyl-3-mercaptopropionate (thiol, 250 μ l) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 4 hours. The solvent was evaporated from the reaction mixture to yield the compound of Formulae I-47, which was further purified by normal phase HPLC using Phenomenex Luna 10u Silica column (25 cmx21.2 mm ID) with a solvent gradient of 10% to 100% Hexane/EtOAc over 24 min, holding at 100% EtOAc for 3 min, at a flow rate of 14.5 ml/min. ELSD was used to monitor the purification process. Compound I-47 (15 mg) eluted at 10.98 min as pure

compound. Compound I-47: UV (Acetonitrile/H₂O) λ_{max} 240 (sh) nm; ESMS m/z 400.1 (M+H)⁺ & 422.1 (M+Na)⁺.

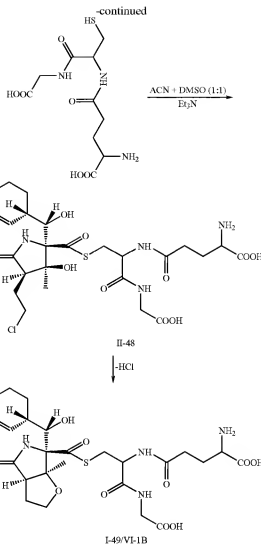
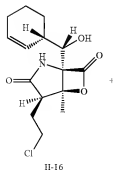




Example 31

Synthesis of the Compound of Formulae I-48 and VI-1B from I-16

[0283] The compound of Formula I-16 (15 mg, 0.048 mmol) was dissolved in 1:1 ratio of ACN/DMSO (8 ml) in a scintillation vial (20 ml) to which triethylamine (40 μ l, 0.29 mmol), Glutathione (44.2 mg, 0.144 mmol) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 3 hours. The solvent was evaporated from the reaction mixture to yield the compound of Formula I-48, which was purified by reversed phase HPLC using Ace 5u C18 column (150 mm \times 22 mm ID) with a solvent gradient of 10% to 70% H₂O/Acetonitrile over 15 min, 70 to 100% Acetonitrile over 5 min, holding at 100% Acetonitrile for 4 min, at a flow rate of 14.5 ml/min. Diode array detector was used to monitor the purification process. Compound I-48 (10 mg) eluted as a pure compound at 8.255 min. Compound I-48: UV (Acetonitrile/H₂O) λ_{max} 235 (sh) nm; ESMS m/z 621.0 (M+H)⁺. Compound I-48 was unstable in solution and converted to compound VI-1B which appeared as a mixture of I-48 and VI-1B in the ratio of 7:3. Compound VI-1B: UV (Acetonitrile/H₂O) λ_{max} 235 (sh) nm; ESMS, m/z 585.2 (M+H)⁺.

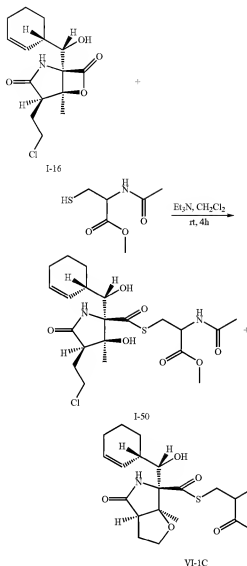


Example 32

Synthesis of the Compound of Formula I-50 and VI-1C from I-16

[0284] The compound of Formula I-16 (10 mg, 0.032 mmol) was dissolved in CH₂Cl₂ (9 ml) in scintillation vial (20 ml) to which triethylamine (26.5 μ l, 0.192 mmol), N-Acetyl-L-Cysteine methyl ester (17 mg, 0.096 mmol) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 4 hours. The solvent was evaporated from the reaction mixture to yield the mixture of compounds of Formulae I-50 and VI-1C, which were further purified by normal phase HPLC using Phenomenex Luna 10u Silica column (25 cm \times 21.2 mm ID) with a solvent gradient of 10% to 100% Hexane/EtOAc over 24 min, holding at 100% EtOAc for 3 min, at a flow rate of 14.5 ml/min. ELSD was used to monitor the purification process. Compounds I-50 (2 mg) and VI-1C (0.2 mg) were eluted at 10.39 and 10.57 min, respectively as pure compounds. Compound I-50: UV (Acetonitrile/H₂O) λ_{max} 230 (sh) nm; ESMS m/z 491.1 (M+H)⁺ & 513.0

(M+Na)⁺. Compound VI-1C: UV (Acetonitrile/H₂O) λ_{max} 215 (sh) nm; ESMS m/z 455.1 (M+H)⁺ & 577.0 (M+Na)⁺



Example 33

Formulation to be Administered Orally or the Like

[0285] A mixture obtained by thoroughly blending 1 g of a compound obtained and purified by the method of the embodiment, 98 g of lactose and 1 g of hydroxypropyl cellulose is formed into granules by any conventional method. The granules are thoroughly dried and sifted to obtain a granule preparation suitable for packaging in bottles or by heat sealing. The resultant granule preparations are orally administered at between approximately 100 ml/day to approximately 1000 ml/day, depending on the symptoms, as deemed appropriate by those of ordinary skill in the art of treating cancerous tumors in humans.

Example 34

Salinosporamide A Inhibits DNA Synthesis and Induces Cytotoxicity of WM Cells

[0286] WM and IgM-secreting cell lines were cultured for 48 hours in the presence of salinosporamide A (Formula I-16) (2.5–40 nM). As shown in FIG. 1A, salinosporamide A inhibited BCWM.1 proliferation, as measured by [³H]-thymidine uptake assay, with an IC₅₀ of 15 nM. salinosporamide A demonstrated similar activity on all cell lines tested, with IC₅₀ between 20 and 30 nM at 48 hours (FIG. 1B).

[0287] The cytotoxic effect of salinosporamide A (2.5–40 nM) on cell lines and WM patient cells by MTT assay was studied. salinosporamide A decreased survival of BCWM.1 cells (IC₅₀ 18 nM; FIG. 1A) and other IgM-secreting cell lines (IC₅₀ 30–40 nM; FIG. 1C). Similarly, salinosporamide A induced cytotoxicity in primary CD19⁺ cells isolated from three patients with WM (IC₅₀ 20–30 nM; FIG. 1D). In contrast, salinosporamide A had no cytotoxic effect on PBMCs from 3 healthy volunteers (data not shown). These results demonstrate that salinosporamide A triggers significant cytotoxicity in tumor cell lines and patient WM cells, without toxicity in normal PBMCs.

Example 35

Salinosporamide A Induces Apoptosis in WM Cells

[0288] The molecular mechanisms whereby salinosporamide A induces cytotoxicity in WM cells was examined. The results demonstrate that salinosporamide A induced dose-dependent apoptosis, as evidenced by Apo2.7 staining in flow cytometry analysis. The percentage of apoptotic BCWM.1 cells increased from 5% (untreated) to 21.2% and 40% after 48 hours of treatment with salinosporamide A 5 nM and 20 nM respectively (FIG. 1E). Similar data were obtained on other IgM secreting cell lines (data not shown).

[0289] Mechanisms whereby salinosporamide A induces apoptosis in WM was determined, and it was demonstrated that salinosporamide A induced caspase-8 and PARP cleavage in a dose-dependent manner (FIG. 1F), without affecting caspase-3 and -9 (data not shown). Moreover salinosporamide A induced down-modulation of the anti-apoptotic protein Mcl-1, with an increased release of the second mitochondria-derived activator of caspases (Smac/DIABLO) from the mitochondria to the cytosol (FIG. 1F). It has been reported that Smac/DIABLO can abrogate the protective effects of inhibitors of apoptosis proteins (IAPs), such as X-linked inhibitor of apoptosis (XIAP).²⁶ Therefore, BCWM.1 cells were treated with salinosporamide A (2.5–20 nM) for 12 hours and demonstrated that salinosporamide A down-regulated the expression of XIAP in a dose-dependent manner, accompanied by an inhibition of other IAPs members, such as c-IAP and survivin (FIG. 1F).

Example 36

Salinosporamide A and Bortezomib Synergistically Induce Cytotoxicity of WM Cells

[0290] Two proteasome inhibitors, salinosporamide A and bortezomib, were investigated to determine whether the combination could be synergistic in inducing cytotoxicity in WM cells. BCWM.1 cells were cultured with salinosporamide A (2.5–10 nM) for 48 hours, in the presence or absence of bortezomib (5–10 nM). salinosporamide A showed a signifi-

cant cytotoxic effect when combined with bortezomib in BCWM.1 cells, as demonstrated using MTT assay at 48 hours (FIG. 2A), salinosporamide A (5 nM) induced cytotoxicity in 12.4% of BCWM.1 cells, which was increased to 39.8% and 69.4% in the presence of bortezomib at 5 nM (Combination Index, CI: 0.72) and 10 nM (CI: 0.6), respectively, indicating synergism. Isobologram analysis, fractions affected and the combination indexes for each of these combinations are summarized in FIG. 2B-C. Similar data were observed on IgM secreting cell lines and primary CD19⁺ cells (data not shown).

[0291] To better define the mechanisms of salinosporamide A/bortezomib-induced cytotoxicity, the effect of salinosporamide A (10 nM), either alone or in combination with bortezomib 10 nM, on BCWM.1 cells using immunoblotting after 12 hours treatment was investigated. It was demonstrated that PARP cleavage was significantly higher using the combination compared to the effect of each agent alone. To further dissect whether apoptosis is mediated through the intrinsic or extrinsic pathways, the effect of salinosporamide A, bortezomib and the combination on caspases-3, 8 and 9 was investigated. As shown in FIG. 2D, it was demonstrated that single agent salinosporamide A induced mild caspase-8 cleavage without affecting caspase-3 and 9 cleavage, while bortezomib induced -9 and -3 cleavage, while the combination of salinosporamide A and bortezomib induced significant caspase-3, -8 and -9 cleavage.

[0292] In addition, the effect of bortezomib and salinosporamide A on Smac and XIAP was investigated and as shown in FIG. 2D, it was demonstrated that the combination of the two proteasome inhibitors induced higher release of Smac/DIABLO and a significant decrease of XIAP, more than the effect of each agent alone. Similarly, it was demonstrated that the IAP member, c-IAP was strongly downregulated by the combination versus single agent treatment (FIG. 2D). In addition, it was shown that salinosporamide A downregulates HSP-27 phosphorylation which in turn leads to increase in the release of Smac/DIABLO from the mitochondria and the induction of caspase-9 cleavage. In parallel it was shown that an upregulation of HSP70, while HSP-90 expression was not modulated (FIG. 2D).

Example 37

Salinosporamide A and Bortezomib Synergistically Inhibit NF- κ B Activation in WM Cells

[0293] The effect of salinosporamide A either alone or in combination with bortezomib on the NF- κ Bp65 DNA binding activity was investigated, studying nuclear extracts from treated cells using the Active Motif assay. It was shown that TNF- α treatment induced NF- κ B recruitment to the nucleus in BCWM.1 cells, which was inhibited by salinosporamide A more than bortezomib, and more significantly by the combination of the two proteasome inhibitors (FIG. 3A). In addition, immunoblotting from nuclear extracts demonstrated that p65 phosphorylation and p50NF- κ B expression were inhibited by salinosporamide A, either alone or in combination with bortezomib, more than bortezomib used as single agent (FIG. 3B). Phospho-p65 translocation from the cytoplasmic compartment to the nucleus was inhibited by the combination of bortezomib and salinosporamide A, resulting in a significant increase in p-p65 expression in the cytoplasmic compartment as shown by immunofluorescence (FIG. 3D). Immunoblotting from nuclear extracts showed that two proteasome inhibitors used in combination inhibited the

expression of p52 and RelB, which are mostly activated through the non-canonical pathway (FIG. 3B).²⁶ Moreover, each agent alone, and more significantly their combination up-regulated the phosphorylation of the inhibitor protein I κ B, as shown in FIG. 3C. Taken together, these data demonstrate that the combination of the two proteasome inhibitors regulate both canonical and non-canonical pathways of NF- κ B in WM.

Example 38

Salinosporamide A and Bortezomib Synergistically Inhibit PI3K/AKT Pathway in WM Cells

[0294] Whether salinosporamide A could affect PI3K/Akt signaling pathway in WM cells was investigated. BCWM.1 cells were treated with increasing doses of salinosporamide A (2.5-20 nM) for 6 hours. As shown in FIG. 4A, salinosporamide A inhibited phosphorylation of Akt (ser473), and downstream GSK3 α/β and ribosomal protein S6 in a dose dependent manner, with no activity on the phosphorylation of the MAP kinase ERK1/2 (thr202/tyr204). The effect of salinosporamide A (10 nM), alone or combined with bortezomib (10 nM), on Akt kinase activity, using an in vitro Akt kinase assay was then investigated. It was shown that salinosporamide A decreased phosphorylation of GSK3 α/β fusion protein, while bortezomib did not modulate Akt phosphorylation. The combination of salinosporamide A and bortezomib showed significant inhibition of Akt activity, indicating a possible mechanism of synergy where salinosporamide A overcomes Akt-dependent bortezomib resistance (FIG. 4B). To further validate the role of the Akt pathway in salinosporamide A-dependent cytotoxicity, an Akt knockdown BCWM.1 cell line established using lentivirus infection was used and it was demonstrated that in the absence of Akt, the cytotoxic effect of salinosporamide A was abrogated (FIG. 4C), indicating that Akt plays an essential role in the cytotoxic activity of salinosporamide A and that this could be an important differential effect between the two proteasome inhibitors and a mechanism of synergy between them in WM.

Example 39

Salinosporamide A Inhibits the Three Proteolytic Activities within the 20S Proteasome

[0295] Cells were treated with salinosporamide A (10 nM) either alone or in combination with bortezomib (10 nM), for 4 hours, and the chymotrypsin-like (CT-L), caspase-like (CL) and trypsin-like (TL) activities were measured using distinct fluorogenic peptides specific for each enzymatic activity.²² As shown in FIG. 4D, bortezomib induced 29%, 5% and 69% reduction of the C-L, T-L and CT-L activities, respectively; compared to salinosporamide A which induced 34.3%, 38.7% and 81% reduction of the C-L, T-L and CT-L activities, respectively. Interestingly, the inhibition of the C-L activity significantly increased to 60% when salinosporamide A and bortezomib were used in combination (FIG. 4D). Similar data were obtained on CD19⁺ primary cells (FIG. 4E-I).

Example 40

The Combination of Salinosporamide A and Bortezomib Overcomes Resistance Induced by the Bone Marrow Microenvironment and IL-6

[0296] Whether salinosporamide A, alone or in combination with bortezomib, inhibits WM cells growth in the context

of the BM milieu was investigated. BCWM.1 cells were cultured with salinosporamide A (2.5–20 nM) and/or bortezomib (10 nM) in the presence or absence of BMSCs, for 48 hours. The viability of BMSCs, assessed by MTT was not affected by salinosporamide A treatment (data not shown). Using [3 H]-TdR uptake assay, adherence of BCWM.1 cells to BMSCs triggered an increase of 55% in proliferation, which was inhibited by salinosporamide A in a dose-dependent manner. This effect was significantly enhanced by the combination with bortezomib (FIG. 5A), confirming that the combination of the two proteasome inhibitors enhanced the anti-tumor activity of each drug used as a single agent, even in presence of BMSCs.

[0297] The effect of the two proteasome inhibitors on cytotoxicity of WM cells in the presence of IL-6 was also investigated. Whether the addition of recombinant IL-6 (25 ng/mL) can overcome the cytotoxic effect of salinosporamide A and bortezomib on WM cells was tested. As shown in FIG. 5B, IL-6 induced proliferation of BCWM.1 cell, and the addition of salinosporamide A (2.5–20 nM), bortezomib (10 nM), or the combination inhibited IL-6-induced proliferation of BCWM.1 cells, indicating that salinosporamide A, alone or more significantly in combination with bortezomib, overcomes resistance induced by IL-6. In addition, IL-6 induced phosphorylation of Akt and STAT-3. salinosporamide A and bortezomib inhibited IL-6-triggered Akt and STAT-3 phosphorylation, which were more significantly down-regulated with the combination of salinosporamide A and bortezomib (FIG. 5C). Whether the two proteasome inhibitors, used as single agent or in combination, could also target non-malignant hematopoietic cells was investigated. It was found that salinosporamide A (10 nM, 20 nM), bortezomib (10 nM) and the combination did not affect the growth of BM hematopoietic progenitor cells as shown using colony-formation assay (FIG. 5D).

Example 41

Salinosporamide A and Bortezomib Inhibit Migration and Adhesion of WM Cells In Vitro and Homing In Vivo

[0298] It was demonstrated that stromal derived factor-1 (SDF-1), one of the important regulators of migration in B-cells, induced migration in BCWM.1 cells at 30 nM SDF-1. To study the effect of salinosporamide A on the migration of WM cells, BCWM.1 cells were incubated with salinosporamide A (10 nM), either alone or in combination with bortezomib (10 nM), for 4 hours (these doses and duration of incubation did not induce apoptosis in WM cells as confirmed by trypan blue and Apo2.7 staining by flow cytometry, data not shown). Cells were then subjected to migration as previously described in Alsayed Y, Ngo H, Runnels J, et al. Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. *Blood*. 2007; 109:2708–2717, which is incorporated by reference in its entirety. salinosporamide A slightly inhibited WM cells migration towards SDF-1, which was significantly inhibited when salinosporamide A was used in combination with bortezomib. In combination with bortezomib induced significant inhibition of BCWM.1 cells migration (FIG. 6A).

[0299] salinosporamide A induced significant inhibition of adhesion to fibronectin when used in combination with bortezomib (FIG. 6B). Therefore, the effect of salinosporamide A and bortezomib on NF- κ B activity in the presence or absence

of fibronectin was examined. As shown in FIG. 6C, FN induced a significant increase in p65 phosphorylation and p50NF- κ B expression, which were inhibited by salinosporamide A, bortezomib, and more significantly by the two drugs in combination.

[0300] DiI-labeled BCWM.1 cells treated with salinosporamide A (10 nM, 4 hours), alone or in combination with bortezomib (5 nM), or DiI-labeled untreated BCWM.1 cells used as control were injected in the tail vein of BALB/c mice, followed by in vivo flow cytometry every 5 minutes for 45 minutes after injection. Neither salinosporamide A nor bortezomib used as single agents significantly inhibited homing of WM cells to the bone marrow, as demonstrated by a rapid decrease of circulating BCWM.1 cells which was observed in the untreated cells as well as on cells treated with salinosporamide A or bortezomib (data not shown). It was demonstrated that pretreatment of BCWM.1 with salinosporamide A in combination with bortezomib resulted in a significant inhibition of homing with a decrease of 45% of cells in the circulation at 45 minutes compared to a decreased of 77% obtained in the untreated cells, suggesting that when the two proteasome inhibitors were used together there is an inhibition in the homing of WM cells to the bone marrow (FIG. 5D).

[0301] The examples described above are set forth solely to assist in the understanding of the embodiments. Thus, those skilled in the art will appreciate that the methods may provide derivatives of compounds.

[0302] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and procedures described herein are presently representative of preferred embodiments and are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention.

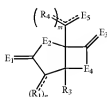
[0303] It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the embodiments disclosed herein without departing from the scope and spirit of the invention.

[0304] All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

[0305] The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions indicates the exclusion of equivalents of the features shown and described or portions thereof. It is recognized that various modifications are possible within the scope of the invention. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed can be resorted to by those skilled in the art, and that such modifications and variations are considered to be falling within the scope of the embodiments of the invention.

What is claimed is:

1. A method of treating Waldenström's Macroglobulinemia comprising administering to an animal a compound having the structure of Formula I, or a pharmaceutically acceptable salt or pro-drug ester thereof:



Formula I

wherein R_1 , R_3 , and R_4 are separately selected from the group consisting of a hydrogen, a halogen, a mono-substituted, a poly-substituted or an unsubstituted variant of the following residues: saturated C_1 - C_{24} alkyl, unsaturated C_2 - C_{24} alkenyl or C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxy, carbonyloxy, aryloxy, carbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy, carbonyl, alkoxy, carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, phenylalkyl, hydroxy, alkylthio, arylthio, oxyalkyl, carboxy, cyano, thio, sulfoxide, sulfone, sulfonate esters, thiocarbonyl, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

wherein m is equal to 1 or 2;

wherein n is equal to 1 or 2;

wherein each of E_1 , E_2 , E_3 , and E_4 is a substituted or unsubstituted heteroatom; and

wherein E_5 is selected from the group consisting of OH , O , OR_{10} , S , SR_{11} , SO_2R_{11} , NH , NH_2 , NOH , NR_{12} , and $NHOR_{13}$.

2. The method of claim 1, wherein R_{10-13} are selected from the group consisting of hydrogen and a substituted or unsubstituted variant of any of the following: C_1 - 24 alkyl, aryl, and heteroaryl.

3. The method of claim 1, wherein the animal is a mammal.

4. The method of claim 1, wherein the animal is a human.

5. The method of claim 1, wherein the animal is a rodent.

6. The method of claim 1, further comprising co-administering a chemotherapeutic agent.

7. The method of claim 6, wherein the chemotherapeutic agent is selected from the group consisting of Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiopeta, Busulfan, Cytosine, Taxol, Taxotere, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincristine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Caminomycin, Aminopterin, Dactinomycin, Mitomycin, Etoposide, Melphalan, tamoxifen and onapristone.

8. The method of claim 6, wherein the chemotherapeutic agent is a proteasome inhibitor.

9. The method of claim 8, wherein the proteasome inhibitor is bortezomib.

10. The method of claim 8, wherein the proteasome inhibitor is carfilzomib.

11. The method of claim 6, wherein the chemotherapeutic agent is a histone deacetylase inhibitor.

12. The method of claim 11, wherein the compound having the structure of Formula I and the histone deacetylase inhibitor work in a synergistic manner to treat Waldenström's Macroglobulinemia.

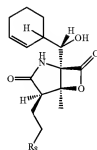
13. The method of claim 11, wherein the histone deacetylase inhibitor is selected from the group consisting of MS-275, APHA compound 8, Apicidin, (-)-Depudecin, sodium butyrate, Scriptaid, Sirtinol, Trichostatin A, Valproic acid (VPA) and Vorinostat (suberoylanilide hydroxamic acid (SAHA)).

14. The method of claim 6, wherein the chemotherapeutic agent is a vascular disrupting agent.

15. The method of claim 14, wherein the vascular disrupting agent is NPI-2358.

16. The method of claim 14, wherein the vascular disrupting agent is combrostatin CA4P.

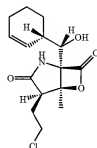
17. A method of treating Waldenström's Macroglobulinemia comprising administering to an animal a compound having the structure of Formula I-15, or a pharmaceutically acceptable salt or pro-drug ester thereof:



I-15

wherein R_8 is hydrogen, fluorine, chlorine, bromine or iodine.

18. The method of claim 17, wherein the compound is:



I-16

19. The method of claim 17, wherein the animal is a mammal.

20. The method of claim 17, wherein the animal is a human.

21. The method of claim 17, wherein the animal is a rodent.

22. The method of claim 17, further comprising co-administering a chemotherapeutic agent.

23. The method of claim 22, wherein the chemotherapeutic agent is selected from the group consisting of Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiopeta, Busulfan, Cytosine, Taxol, Taxotere, Methotrexate, Cisplatin, Melphalan, Vin-

blastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincristine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Caminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins, Melphalan, tamoxifen and onapristone.

24. The method of claim 22, wherein the chemotherapeutic agent is a proteasome inhibitor.

25. The method of claim 24, wherein the proteasome inhibitor is bortezomib.

26. The method of claim 24, wherein the proteasome inhibitor is carfilzomib.

27. The method of claim 22, wherein the chemotherapeutic agent is a histone deacetylase inhibitor.

28. The method of claim 27, wherein the compound having the structure of Formula I and the histone deacetylase inhibitor work in a synergistic manner to treat Waldenström's Macroglobulinemia.

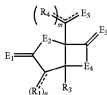
29. The method of claim 27, wherein the histone deacetylase inhibitor is selected from the group consisting of MS-275, APLA compound 8, Apicidin, (-)-Depudecin, sodium butyrate, Scriptaid, Sirtinol, Trichostatin A, Valproic acid (VPA) and Vorinostat (suberoylanilide hydroxamic acid (SAHA)).

30. The method of claim 22, wherein the chemotherapeutic agent is a vascular disrupting agent.

31. The method of claim 30, wherein the vascular disrupting agent is NPI-2358.

32. The method of claim 30, wherein the vascular disrupting agent is combrestatin CA4P.

33. A method of inducing the growth of a Waldenström's Macroglobulinemia cell comprising contacting the cell with a compound having the structure of Formula I, or a pharmaceutically acceptable salt or pro-drug ester thereof:



Formula I

wherein R₁, R₃, and R₄ are separately selected from the group consisting of a hydrogen, a halogen, a mono-substituted, a poly-substituted or an unsubstituted variant of the following residues: saturated C₁-C₂₄ alkyl, unsaturated C₂-C₂₄ alkenyl or C₂-C₂₄ alkynyl, acyl, acyloxy, alkoxy, alkoxyalkoxy, aryloxy, aryloxyalkoxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy, carbonyl, alkoxy carbonyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, phenylalkoxy, hydroxy, alkylthio, arylthio, oxyalkyl, carboxy, cyano, thio, sulfoxide, sulfone, sulfonate esters, thiocarbonyl, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

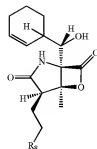
wherein m is equal to 1 or 2;

wherein n is equal to 1 or 2;

wherein each of E₁, E₂, E₃, and E₄ is a substituted or unsubstituted heteroatom; and

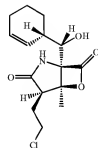
wherein E₅ is selected from the group consisting of OH, O, OR₁₀, S, SR₁₁, SO₂R₁₁, NH, NH₂, NOH, NHOH, NR₁₂, and NHOR₁₃.

34. A method of inhibiting the growth of a Waldenström's Macroglobulinemia cell comprising contacting the cell with a compound having the structure of Formula I-15, or a pharmaceutically acceptable salt or pro-drug ester thereof:



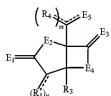
wherein R₈ is hydrogen, fluorine, chlorine, bromine or iodine.

35. The method of claim 34, wherein the compound is:



II-16

36. A method of inducing apoptosis of a Waldenström's Macroglobulinemia cell comprising contacting the cell with a compound having the structure of Formula I, and a pharmaceutically acceptable salt or pro-drug ester thereof:



Formula I

wherein R₁, R₃, and R₄ are separately selected from the group consisting of a hydrogen, a halogen, a mono-substituted, a poly-substituted or an unsubstituted variant of the following residues: saturated C₁-C₂₄ alkyl, unsaturated C₂-C₂₄ alkenyl or C₂-C₂₄ alkynyl, acyl, acyloxy, alkoxy, alkoxyalkoxy, aryloxy, aryloxyalkoxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy, carbonyl, alkoxy carbonyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkyl, hydroxy, alkylthio, arylthio, oxyalkyl, carboxy, cyano, thio, sulfoxide, sulfone, sulfonate esters, thiocarbonyl, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

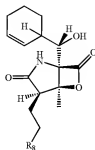
wherein m is equal to 1 or 2;

wherein n is equal to 1 or 2;

wherein each of E₁, E₂, E₃, and E₄ is a substituted or unsubstituted heteroatom; and

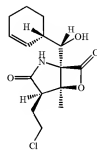
wherein E₅ is selected from the group consisting of OH, O, OR₁₀, S, SR₁₁, SO₂R₁₁, NH, NH₂, NOH, NHOH, NR₁₂, and NHOR₁₃.

37. A method of inducing apoptosis of a Waldenstrom's Macroglobulinemia cell comprising contacting the cell with a compound having the structure of Formula I-15 and a pharmaceutically acceptable salt or pro-drug ester thereof:



wherein R_8 is hydrogen, fluorine, chlorine, bromine or iodine.

38. The method of claim 37, wherein the compound is:



I-16

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CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE,
EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID,
IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC,
LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN,
MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH,
PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV,
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[Continued on next page]

(54) Title: A METHOD OF USING PROTEASOME INHIBITORS IN COMBINATION WITH HISTONE DEACETYLASE INHIBITORS TO TREAT CANCER

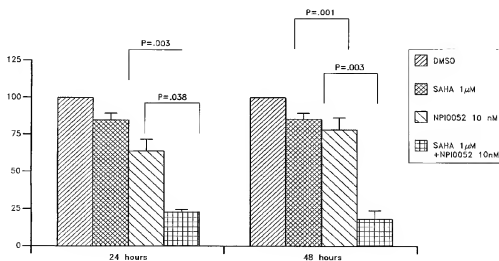


FIG. 9

(57) Abstract: Disclosed are methods of treating cancer comprising administering to the animal, a therapeutically effective amount of proteasome inhibitors and one or more histone deacetylase inhibitor. The animal is a mammal, preferably a human or a rodent.



NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG,
CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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A METHOD OF USING PROTEASOME INHIBITORS IN COMBINATION WITH HISTONE DEACETYLASE INHIBITORS TO TREAT CANCER

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to certain compounds and to methods for the preparation and the use of certain compounds in the fields of chemistry and medicine.

Description of the Related Art

[0002] Cancer is a leading cause of death in the United States. Despite significant efforts to find new approaches for treating cancer, the primary treatment options remain surgery, chemotherapy and radiation therapy, either alone or in combination. Surgery and radiation therapy, however, are generally useful only for fairly defined types of cancer, and are of limited use for treating patients with disseminated disease. Chemotherapy is the method that is generally useful in treating patients with metastatic cancer or diffuse cancers such as leukemias. Although chemotherapy can provide a therapeutic benefit, it often fails to result in cure of the disease due to the patient's cancer cells becoming resistant to the chemotherapeutic agent. Due, in part, to the likelihood of cancer cells becoming resistant to a chemotherapeutic agent, such agents are commonly used in combination to treat patients.

[0003] Similarly, infectious diseases caused, for example, by bacteria, fungi and protozoa are becoming increasingly difficult to treat and cure. For example, more and more bacteria, fungi and protozoa are developing resistance to current antibiotics and chemotherapeutic agents. Examples of such microbes include *Bacillus*, *Leishmania*, *Plasmodium* and *Trypanosoma*.

[0004] Furthermore, a growing number of diseases and medical conditions are classified as inflammatory diseases. Such diseases include conditions such as asthma to cardiovascular diseases. These diseases continue to affect larger and larger numbers of people worldwide despite new therapies and medical advances.

[0005] Therefore, a need exists for additional chemotherapeutics, anti-microbial agents, and anti-inflammatory agents to treat cancer, inflammatory diseases and infectious

disease. A continuing effort is being made by individual investigators, academia and companies to identify new, potentially useful chemotherapeutic and anti-microbial agents.

[0006] Marine-derived natural products are a rich source of potential new anti-cancer agents and anti-microbial agents. The oceans are massively complex and house a diverse assemblage of microbes that occur in environments of extreme variations in pressure, salinity, and temperature. Marine microorganisms have therefore developed unique metabolic and physiological capabilities that not only ensure survival in extreme and varied habitats, but also offer the potential to produce metabolites that would not be observed from terrestrial microorganisms (Okami, Y. 1993 *J Mar Biotechnol* 1:59). Representative structural classes of such metabolites include terpenes, peptides, polyketides, and compounds with mixed biosynthetic origins. Many of these molecules have demonstrable anti-tumor, anti-bacterial, anti-fungal, anti-inflammatory or immunosuppressive activities (Bull, A.T. *et al.* 2000 *Microbiol Mol Biol Rev* 64:573; Cragg, G.M. & D.J. Newman 2002 *Trends Pharmacol Sci* 23:404; Kerr, R.G. & S.S. Kerr 1999 *Exp Opin Ther Patents* 9:1207; Moore, B.S 1999 *Nat Prod Rep* 16:653; Faulkner, D.J. 2001 *Nat Prod Rep* 18:1; Mayer, A. M. & V.K. Lehmann 2001 *Anticancer Res* 21:2489), validating the utility of this source for isolating invaluable therapeutic agents. Further, the isolation of novel anti-cancer and anti-microbial agents that represent alternative mechanistic classes to those currently on the market will help to address resistance concerns, including any mechanism-based resistance that may have been engineered into pathogens for bioterrorism purposes.

Summary of the Invention

[0007] The embodiments disclosed herein generally relate to chemical compounds, including heterocyclic compounds and analogs thereof. Some embodiments are directed to the use of compounds as proteasome inhibitors.

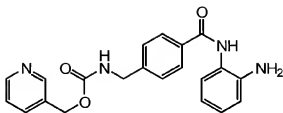
[0008] In other embodiments, the compounds are used to treat neoplastic diseases, for example, to inhibit the growth of tumors, cancers and other neoplastic tissues. The methods of treatment disclosed herein can be employed with any patient suspected of carrying tumorous growths, cancers, or other neoplastic growths, either benign or malignant ("tumor" or "tumors" as used herein encompasses tumors, cancers, disseminated neoplastic

cells and localized neoplastic growths). Examples of such growths include but are not limited to breast cancers; hematologic malignancies including lymphomas, such as Hodgkin's lymphoma and non-hodgkin's lymphoma, leukemias, and multiple myelomas; osteosarcomas, angiosarcomas, fibrosarcomas and other sarcomas; leukemias; sinus tumors; ovarian, ureteral, bladder, prostate and other genitourinary cancers; colon, esophageal and stomach cancers and other gastrointestinal cancers; rectal cancers; lung cancers (such as large cell carcinoma, small cell carcinoma, large cell carcinoma, squamous cell carcinoma, adenocarcinoma, and bronchioloalveolar carcinoma); lymphomas; myelomas; teratomas; pancreatic cancers; liver cancers; kidney cancers; endocrine cancers; skin cancers; eye cancers; cervical cancers; anal carcinomas; hepatocellular carcinomas; laryngeal cancers; renal cell carcinomas; testicular cancers; thyroid cancers; osteosarcomas; chondrosarcoma; Kaposi's sarcoma; rhabdomyosarcomas; melanomas; angiomas; and brain or central nervous system (CNS; glioma) cancers. In general, the tumor or growth to be treated can be any tumor or cancer, primary or secondary. Certain embodiments relate to methods of treating neoplastic diseases in animals. The method can include, for example, administering an effective amount of a compound to a patient in need thereof. Other embodiments relate to the use of compounds in the manufacture of a pharmaceutical or medicament for the treatment of a neoplastic disease.

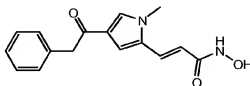
[0009] The compounds can be administered or used in combination with treatments such as chemotherapy, radiation, and biologic therapies. In some embodiments the compounds can be administered or used with a chemotherapeutic agent. Examples of such chemotherapeutics include Alkaloids, alkylating agents, antibiotics, antimetabolites, enzymes, hormones, platinum compounds, immunotherapeutics (antibodies, T-cells, epitopes), BRMs, and the like. Examples include, Vincristine, Vinblastine, Vindesine, Paclitaxel (Taxol), Docetaxel, topoisomerase inhibitors epipodophyllotoxins (Etoposide (VP-16), Teniposide (VM-26)), Camptothecin, nitrogen mustards (cyclophosphamide), Nitrosoureas, Carmustine, lomustine, dacarbazine, hydroxymethylmelamine, thiotepa and mitocycin C, Dactinomycin (Actinomycin D), anthracycline antibiotics (Daunorubicin, Daunomycin, Cerubidine), Doxorubicin (Adriamycin), Idarubicin (Idamycin), Anthracenediones (Mitoxantrone), Bleomycin (Blenoxane), Plicamycin (Mithramycin, Antifolates (Methotrexate (Folex, Mexate)), purine antimetabolites (6-mercaptopurine (6-

MP, Purinethol) and 6- thioguanine (6-TG). The two major anticancer drugs in this category are 6-mercaptopurine and 6-thioguanine, Chlorodeoxyadenosine and Pentostatin, Pentostatin (2'-deoxycoformycin), pyrimidine antagonists, Avastin, Leucovorin, Oxaliplatin, fluoropyrimidines (5-fluorouracil(Adrucil), 5-fluorodeoxyuridine (FdUrd) (Floxuridine)), Cytosine Arabinoside (Cytosar, ara-C), Fludarabine, Hydroxyurea, glucocorticoids, antiestrogens, tamoxifen, nonsteroidal antiandrogens, flutamide, aromatase inhibitors Anastrozole(Arimidex), Cisplatin, 6-Mercaptopurine and Thioguanine, Methotrexate, Cytosan, Cytarabine, L-Asparaginase, Steroids: Prednisone and Dexamethasone. Also, proteasome inhibitors such as bortezomib can be used in combination with the instant compounds, for example. Examples of biologics can include agents such as TRAIL antibodies to TRAIL, integrins such as alpha-V-beta-3 ($\alpha V\beta 3$) and / or other cytokine/growth factors that are involved in angiogenesis, VEGF, EGF, FGF and PDGF. In some aspects, the compounds can be conjugated to or delivered with an antibody. The above-described combination methods can be used to treat a variety of conditions, including cancer and neoplastic diseases, inflammation, and microbial infections.

[0010] In some embodiments, the compounds are administered in combination with a histone deacetylase inhibitor (HDACi). In various embodiments, the HDACi is selected from the group consisting of:

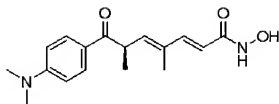


(pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate
(MS-275 or SNDX-275),

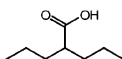


APHA compound 8,

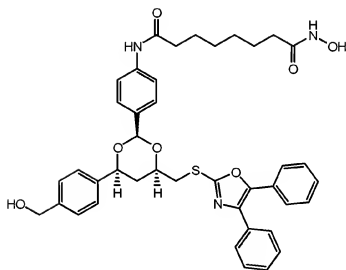
Sirtinol,



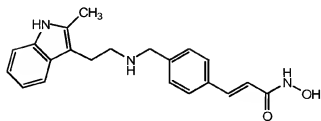
trichostatin A,



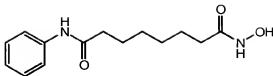
valproic acid,



tubacin,



panobinostat, and

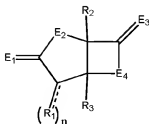


vorinostat (suberoylanilide hydroxamic acid (SAHA)).

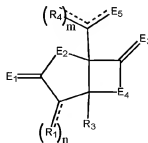
[0011] In still other embodiments, the compounds are used to treat inflammatory conditions. Certain embodiments relate to methods of treating inflammatory conditions in animals. The method can include, for example, administering an effective amount of a compound to a patient in need thereof. Other embodiments relate to the use of compounds in the manufacture of a pharmaceutical or medicament for the treatment of inflammation.

[0012] In certain embodiments, the compounds are used to treat infectious diseases. The infectious agent can be a microbe, for example, bacteria, fungi, protozoans, and microscopic algae, or viruses. Further, the infectious agent can be *B. anthracis* (anthrax). In some embodiments the infectious agent is a parasite. For example, the infectious agent can be *Plasmodium*, *Leishmania*, and *Trypanosoma*. Certain embodiments relate to methods of treating infectious agents in animals. The method can include, for example, administering an effective amount of a compound to a patient in need thereof. Other embodiments relate to the use of compounds in the manufacture of a pharmaceutical or medicament for the treatment of infectious agents.

[0013] The present embodiments provide methods of treating cancer comprising administering to an animal a compound having the structure of any one of Formulas I and II, or a pharmaceutically acceptable salt or pro-drug thereof:



Formula I



Formula II

[0014] in combination with a histone deacetylase inhibitor (HDACi);

[0015] wherein:

[0016] the dashed lines represent a single or a double bond,

[0017] each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxyacarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0018] n is 1 or 2, where if n is 2, then each R_1 can be the same or different;

[0019] m is 1 or 2, where if m is 2, then each R_4 can be the same or different;

[0020] R_2 is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxyacarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0021] R_3 is a halogen or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxyacarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

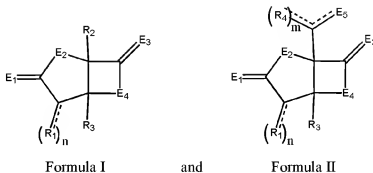
[0022] each of E_1 , E_3 , E_4 and E_5 is an optionally substituted heteroatom;

[0023] E_2 is an optionally substituted heteroatom or $-CH_2-$ group; and

[0024] each R_4 is separately a halogen, a cyano, a nitro, an azido, or a thiocyno, or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxyacarbonylacyl,

amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

[0025] The present embodiments provide pharmaceutical compositions comprising a histone deacetylase inhibitor (HDACi) and a compound of any one of Formulas I and II:



[0026] wherein:

[0027] the dashed lines represent a single or a double bond;

[0028] each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyanate, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0029] n is 1 or 2, where if n is 2, then each R_1 can be the same or different;

[0030] m is 1 or 2, where if m is 2, then each R_4 can be the same or different;

[0031] R_2 is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyanate, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl,

alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0032] R_3 is a halogen or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

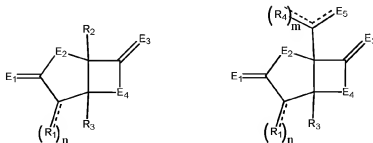
[0033] each of E_1 , E_3 , E_4 and E_5 is an optionally substituted heteroatom;

[0034] E_2 is an optionally substituted heteroatom or $-CH_2-$ group; and

[0035] each R_4 is separately a halogen, a cyano, a nitro, an azido, or a thiocyanato, or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

[0036] Other embodiments relate to methods of treating a neoplastic disease in an animal. The methods can include, for example, administering to the animal, a therapeutically effective amount of a compound of a formula selected from Formulae I and II, and pharmaceutically acceptable salts and pro-drug esters thereof.

[0037] The present embodiments provide methods of inhibiting the growth of a cancer cell comprising contacting the cell with a HDACi and a compound having the structure of any one of Formulas I and II:



Formula I

and

Formula II

[0038] wherein:**[0039]** the dashed lines represent a single or a double bond;

[0040] each **R₁** is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxyacarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0041] **n** is 1 or 2, where if **n** is 2, then each **R₁** can be the same or different;**[0042]** **m** is 1 or 2, where if **m** is 2, then each **R₄** can be the same or different;

[0043] **R₂** is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxyacarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

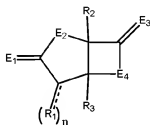
[0044] **R₃** is a halogen or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxyacarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

[0045] each of **E₁**, **E₃**, **E₄** and **E₅** is an optionally substituted heteroatom;**[0046]** **E₂** is an optionally substituted heteroatom or -CH₂- group; and

[0047] each **R₄** is separately a halogen, a cyano, a nitro, an azido, or a thiocyno, or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl,

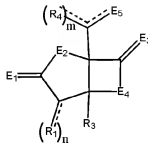
cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

[0048] The present embodiments provide methods of inducing apoptosis of a cancer cell comprising contacting the cell with a HDACi and a compound having the structure of any one of Formulas I and II:



Formula I

and



Formula II

[0049] wherein:

[0050] the dashed lines represent a single or a double bond;

[0051] each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkylloxycarbonyloxy, arylloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0052] n is 1 or 2, where if n is 2, then each R_1 can be the same or different;

[0053] m is 1 or 2, where if m is 2, then each R_4 can be the same or different;

[0054] R_2 is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkylloxycarbonyloxy, arylloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl,

alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0055] R_3 is a halogen or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

[0056] each of E_1 , E_3 , E_4 and E_5 is an optionally substituted heteroatom;

[0057] E_2 is an optionally substituted heteroatom or $-CH_2-$ group; and

[0058] each R_4 is separately a halogen, a cyano, a nitro, an azido, or a thiocycano, or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

Brief Description of the Drawings

[0059] The accompanying drawings, which are incorporated in and form part of the specification, merely illustrate certain preferred embodiments of the present invention. Together with the remainder of the specification, they are meant to serve to explain preferred modes of making certain compounds of the invention to those of skilled in the art. In the drawings:

[0060] Fig. 1 shows Salinosporamide A (NPI-0052) inhibits the proteolytic activities of the 20S proteasome in leukemia cell lines.

[0061] Fig. 2 shows *in vitro* and *in vivo* effects of Salinosporamide A (NPI-0052).

[0062] Fig. 3 shows Salinosporamide A (NPI-0052) induced apoptosis through caspase activation and mitochondrial perturbations.

[0063] Fig. 4 shows the role of caspase-8 and FADD in Salinosporamide A (NPI-0052) induced apoptosis.

[0064] Fig. 5 shows the free radical scavenger, NAC, protects from Salinosporamide A (NPI-0052) induced apoptosis.

[0065] Fig. 6 shows low doses of Salinosporamide A (NPI-0052) and HDAC inhibitors induce synergistic apoptosis.

[0066] Fig. 7 shows schematic representation of the mechanism of action of Salinosporamide A (NPI-0052).

[0067] Fig. 8 shows the effect of the combination of MS-275 with bortezomib and the combination of MS-275 with Salinosporamide A to induce synergistic apoptosis.

[0068] Fig. 9 shows that Salinosporamide A (10 nM) enhances the activity of vorinostat (SAHA, 1 μ M) in Hodgkin's HD-LM2 Lymphoma cell lines.

[0069] Fig. 10 shows that Salinosporamide A (50 nM) enhances the activity of vorinostat (SAHA, 5 μ M) in Hodgkin's L428 cells lines and that Salinosporamide A (50 nM) enhances the activity of vorinostat (SAHA, 5 μ M) in KM-H2 cell lines.

[0070] Fig. 11 shows the effect of combinations of Salinosporamide A (14 nM) with MS-275 (0.5 μ M, 2 μ M and 3 μ M) in a human myeloma cell line (RPMI 8226 MM cells), the effect of combinations of Salinosporamide A (18 nM) with MS-275 (1 μ M) and the effect of combinations of Salinosporamide A (16 nM) with MS-275 (1.5 μ M).

[0071] Fig. 12 shows the effect of combinations of Salinosporamide A (12 nM, 14 nM and 18 nM) with MS-275 (1.5 μ M) in a human myeloma cell line (OPM-1 MM cells), and the effect of combinations of Salinosporamide A (18 nM and 20 nM) with MS-275 (2 μ M) in a human myeloma cell line (OPM-1 MM cells).

[0072] Fig. 13 shows the effect of combinations of Salinosporamide A (16 nM and 18 nM) with MS-275 (0.5 μ M, 1 μ M, 1.5 μ M and 2 μ M) in a human myeloma cell line (DHL-6 MM cells).

[0073] Fig. 14 shows the effect of combinations of Salinosporamide A (12 nM, 14 nM, and 20 nM) with MS-275 (0.5 μ M, 1 μ M, 1.5 μ M and 2 μ M) in a human myeloma cell line (Dox-6 MM cells).

[0074] Fig. 15 shows the effect of combinations of Salinosporamide A (14 nM, 16 nM, and 20 nM) with MS-275 (0.5 μ M, 1 μ M, 1.5 μ M and 2 μ M) in a human myeloma cell line (Dox-40 MM cells).

[0075] Fig. 16 shows the effect of combinations of Salinosporamide A (12 nM, 14 nM, 18 nM and 20 nM) with MS-275 (0.5 μ M, 1 μ M, and 2 μ M) in a human myeloma cell line (LR-5 MM cells).

[0076] Fig. 17 shows the effect of combinations of Salinosporamide A (3 nM, 5 nM, and 7 nM) with MS-275 (0.25 μ M, 0.5 μ M, and 1 μ M) in a human myeloma cell line (MM.1R cells).

[0077] Fig. 18A shows activity of Salinosporamide A (10 nM) and vorinostat (5 μ M) alone and in combination on SB-2 line.

[0078] Fig. 18B shows activity of Salinosporamide A (10 nM) and vorinostat (5 μ M) alone and in combination on WM-266-4 melanoma cell line.

[0079] Fig. 19 shows activity of Salinosporamide A (NPI-0052, 10 nM) and vorinostat (SAHA, 5 μ M) alone and in combination on MeWo melanoma cell lines.

[0080] Fig. 20 shows growth inhibition effects of Salinosporamide A (NPI-0052) at doses of 5 nM to 500 nM as single agent or in combination with Vorinostat (SAHA, 2 μ M) in various lung cancer cell lines.

[0081] Fig. 21 shows growth inhibition effects of Salinosporamide A (NPI-0052) at doses of 5 nM to 500 nM as single agent or in combination with Vorinostat (2 μ M) in various lung cancer cell lines.

[0082] Fig. 22A-H shows isobologram analyses for eight lung carcinoma cell lines treated with Salinosporamide A (NPI-0052) and vorinostat (SAHA).

[0083] Fig. 23 shows that human pancreatic carcinoma cell lines are resistant to treatment with gemcitabine alone, when gemcitabine (1 μ M or 10 μ M) is used in combination with Salinosporamide A a dosage effect is observed.

[0084] Fig. 24 shows the effect of Salinosporamide A (NPI-0052) with vorinostat (SAHA) increases apoptosis compared to treatment with the individual agents alone.

[0085] Fig. 25 shows HDAC inhibitor MS-275 can decrease mRNA expression of 20S proteasome β subunits in Jurkat cells.

[0086] Fig. 26 shows vorinostat can decrease mRNA expression of 20S proteasomal $\beta 5$ subunit in Jurkat cells as measured at 12 hours and 18 hours. The expression of $\beta 5$ mRNA was analyzed by real time PCR.

[0087] Fig. 27 shows MS-275 in combination with Salinosporamide A or bortezomib causes Histone-3 to hyperacetylate in Jurkat T-cells.

[0088] Fig. 28 shows combination of Salinosporamide A with vorinostat causes hyperacetylation of Histone-3 in Jurkat cells, the addition of N-acetylcysteine (NAC) inhibits the hyperacetylation of Histone-3.

[0089] Fig. 29 shows histone-3 ubiquitination is not affected by Salinosporamide A (NPI-0052) or the combination of Salinosporamide A (NPI-0052) with MS-275.

[0090] Fig. 30 shows effects of vorinostat pretreatment of human Jurkat ALL cells followed by treatment with bortezomib.

[0091] Fig. 31 shows effects of simultaneous treatment of human Jurkat ALL cells with vorinostat and bortezomib.

[0092] Fig. 32 shows effects of vorinostat pretreatment of human Jurkat ALL cells followed by treatment with Salinosporamide A.

[0093] Fig. 33 shows effects of simultaneous treatment of human Jurkat ALL cells with vorinostat and Salinosporamide A.

[0094] Fig. 34 shows effect of MS-275 and Salinosporamide A in combination and alone on superoxide levels in Jurkat T-cells.

[0095] Fig. 35 shows effect of MS-275 and Salinosporamide A in combination and alone on superoxide levels in Caspase-8 deficient cells.

[0096] Fig. 36 shows vorinostat in combination with Salinosporamide A or bortezomib causes formation of reactive oxygen species (ROS), when N-acetylcysteine (NAC) is included in the combinations the amount of ROS decrease as measured by mean fluorescence.

[0097] Fig. 37 shows N-acetylcysteine (NAC) decreases formation of ROS when combined with vorinostat (Zolinza) and Salinosporamide A but not when combined with vorinostat and bortezomib (Velcade).

[0098] Fig. 38 shows combination of vorinostat (SAHA) and Salinosporamide A (NPI) does not induce apoptosis in cells that are caspase-8 deficient as strongly as cells that are not caspase-8 deficient.

[0099] Fig. 39 shows regulation of NF- κ B Activity *in vitro* by vorinostat and Salinosporamide A alone and in combination.

[0100] Fig. 40 shows regulation of NF- κ B Activity *in vitro* in human pancreatic carcinoma cells by vorinostat and Salinosporamide A alone and in combination.

[0101] Fig. 41 shows combination of Salinosporamide A and vorinostat exhibit enhanced activity in an orthotopic pancreatic tumor model.

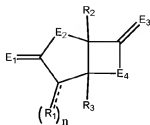
Detailed Description of the Preferred Embodiment

[0102] Numerous references are cited herein. The references cited herein, including the U.S. patents cited herein, are each to be considered incorporated by reference in their entirety into this specification.

[0103] Mono-therapy with single chemotherapeutic agents and targeted compounds is rarely able to surmount the divergent multi-pathway survival and growth signaling pathways that are critical to the survival of cancer cells. As a result, researchers and clinicians have resorted to investigating the potential of combination therapies. To accurately predict the best potential combinations it is therefore necessary to identify the specific aberrant signaling pathways which are responsible for a cancer phenotype.

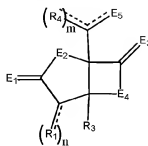
[0104] By “co-administration” or use “in combination,” it is meant that the two or more agents may be found in the patient’s bloodstream at the same time, regardless of when or how they are actually administered. In one embodiment, the agents are administered simultaneously. In one such embodiment, administration in combination is accomplished by combining the agents in a single dosage form. In another embodiment, the agents are administered sequentially. In one embodiment the agents are administered through the same route, such as orally. In another embodiment, the agents are administered through different routes, such as one being administered orally and another being administered i.v. In one advantageous embodiment, the pharmacokinetics of the two or more agents are substantially the same.

[0105] Some embodiments provide a method of treating cancer comprising administering to an animal a compound having the structure of any one of Formulas I and II, or a pharmaceutically acceptable salt or pro-drug ester thereof:



Formula I

and



Formula II

[0106] in combination with a histone deacetylase inhibitor (HDACi);

[0107] wherein:

[0108] the dashed lines represent a single or a double bond;

[0109] each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyanate, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0110] n is 1 or 2, where if n is 2, then each R_1 can be the same or different;

[0111] m is 1 or 2, where if m is 2, then each R_4 can be the same or different;

[0112] R_2 is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyanate, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0113] R_3 is a halogen or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

[0114] each of E_1 , E_3 , E_4 and E_5 is an optionally substituted heteroatom;

[0115] E_2 is an optionally substituted heteroatom or $-CH_2-$ group; and

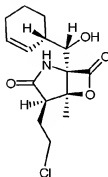
[0116] each R_4 is separately a halogen, a cyano, a nitro, an azido, or a thioccyano, or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

[0117] In some embodiments E_5 can be, for example, OH, O, OR_{10} , S, SR_{11} , SO_2R_{11} , NH, NH_2 , NOH, $NHOH$, NR_{12} , and $NHOR_{13}$, wherein R_{10-13} may separately include, for example, hydrogen, a substituted or unsubstituted of any of the following: alkyl, an aryl, a heteroaryl, and the like. R_3 can be methyl. Furthermore, R_4 may include a cyclohexyl. Also, each of E_1 , E_3 and E_4 can be O and E_2 can be NH. Preferably, R_1 can be CH_2CH_2X , wherein X is selected from the group consisting of hydrogen, fluorine, chlorine, bromine, and iodine; wherein R_4 may include a cyclohexyl; wherein R_3 can be methyl; and wherein each of E_1 , E_3 and E_4 separately can be O and E_2 can be NH. In some embodiments, R_1 can be alkyl optionally substituted with a boronic ester or boronic ester. For example, the boronic ester can be $B(OMethyl)_2$, $B(OEthyl)_2$, $B(OPropyl)_2$, $B(OPhenyl)_2$, and the like.

[0118] In certain embodiments, the cancer can be selected from the group consisting of breast cancer, sarcoma, leukemia, uretal cancer, bladder cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, brain cancer and central nervous system (CNS) cancer. In a typical embodiment, the cancer can be leukemia, lymphoma, and the like. In

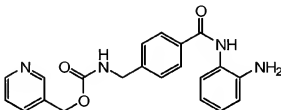
certain embodiments the cancer can comprise a tumor. In a typical embodiment, the tumor can be a refractory solid tumor. In certain embodiments the method can further comprise co-administering a chemotherapeutic agent.

[0119] In certain embodiments, the compound is Salinosporamide A;

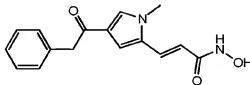


Salinosporamide A

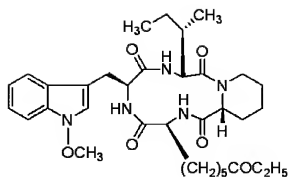
[0120] In certain embodiments, the HDACi and the compound having the structure of any one of Formulas I and II can work in a synergistic manner to treat cancer. In certain embodiments, the HDACi can be selected from the group consisting of:



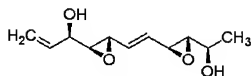
(pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate,



APHA compound 8,



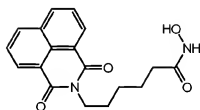
apicidin,



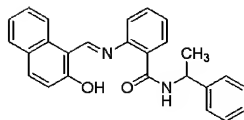
(-)-Depudecin,



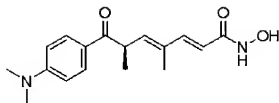
sodium Butyrate,



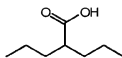
Scriptaid,



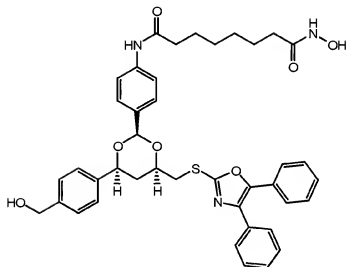
Sirtinol,



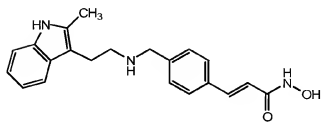
trichostatin A,



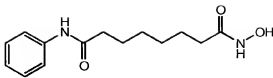
valproic acid,



tubacin,



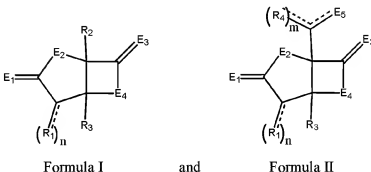
panobinostat, and



vorinostat (suberoylanilide hydroxamic acid (SAHA)).

[0121] For example, the HDACi can be (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275), valproic acid, vorinostat, and the like.

[0122] Some embodiments provide a pharmaceutical composition comprising a histone deacetylase inhibitor (HDACi) and a compound of any one of Formulas I and II:



[0123] wherein:

[0124] the dashed lines represent a single or a double bond;

[0125] each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0126] n is 1 or 2, where if n is 2, then each R_1 can be the same or different;

[0127] m is 1 or 2, where if m is 2, then each R_2 can be the same or different;

[0128] R_2 is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl,

alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0129] R_3 is a halogen or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

[0130] each of E_1 , E_3 , E_4 and E_5 is an optionally substituted heteroatom;

[0131] E_2 is an optionally substituted heteroatom or $-CH_2-$ group; and

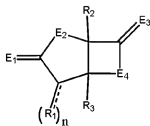
[0132] each R_4 is separately a halogen, a cyano, a nitro, an azido, or a thiocyno, or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

[0133] In some embodiments E_5 can be, for example, OH, O, OR_{10} , S, SR_{11} , SO_2R_{11} , NH, NH_2 , NOH, $NHOH$, NR_{12} , and $NHOR_{13}$, wherein R_{10-13} may separately include, for example, hydrogen, a substituted or unsubstituted of any of the following: alkyl, an aryl, a heteroaryl, and the like. R_3 can be methyl. Furthermore, R_4 may include a cyclohexyl. Also, each of E_1 , E_3 and E_4 can be O and E_2 can be NH. Preferably, R_1 can be CH_2CH_2X , wherein X is selected from the group consisting of hydrogen, fluorine, chlorine, bromine, and iodine; wherein R_4 may include a cyclohexyl; wherein R_3 can be methyl; and wherein each of E_1 , E_3 and E_4 separately can be O and E_2 can be NH. In some embodiments, R_1 can be alkyl optionally substituted with a boronic ester or boronic ester. For example, the boronic ester can be $B(OMethyl)_2$, $B(OEthyl)_2$, $B(OPropyl)_2$, $B(OPhenyl)_2$, and the like.

[0134] In a typical embodiment the compound is Salinosporamide A. In certain embodiments the HDACi can be selected from the group consisting of (pyridin-3-yl)methyl

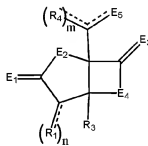
4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275), APIA compound 8, Apicidin, (-)-Depudecin, sodium butyrate, scriptaid, sirtinol, trichostatin A, valproic acid, vorinostat and the like. For example, the HDACi can be (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275), HDACi is valproic acid, vorinostat, and the like.

[0135] Some embodiments provide a method of inhibiting the growth of a cancer cell comprising contacting the cell with a HDACi and a compound having the structure of any one of Formulas I and II:



Formula I

and



Formula II

[0136] wherein:

[0137] the dashed lines represent a single or a double bond;

[0138] each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxyacarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0139] n is 1 or 2, where if n is 2, then each R_1 can be the same or different;

[0140] m is 1 or 2, where if m is 2, then each R_4 can be the same or different;

[0141] R_2 is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl,

alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0142] R_3 is a halogen or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

[0143] each of E_1 , E_3 , E_4 and E_5 is an optionally substituted heteroatom;

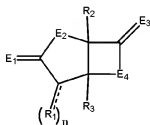
[0144] E_2 is an optionally substituted heteroatom or $-CH_2-$ group; and

[0145] each R_4 is separately a halogen, a cyano, a nitro, an azido, or a thiocyno, or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

[0146] In some embodiments E_5 can be, for example, OH, O, OR_{10} , S, SR_{11} , SO_2R_{11} , NH, NH_2 , NOH, $NHOH$, NR_{12} , and $NHOR_{13}$, wherein R_{10-13} may separately include, for example, hydrogen, a substituted or unsubstituted of any of the following: alkyl, an aryl, a heteroaryl, and the like. R_3 can be methyl. Furthermore, R_4 may include a cyclohexyl. Also, each of E_1 , E_3 and E_4 can be O and E_2 can be NH. Preferably, R_1 can be CH_2CH_2X , wherein X is selected from the group consisting of hydrogen, fluorine, chlorine, bromine, and iodine; wherein R_4 may include a cyclohexyl; wherein R_3 can be methyl; and wherein each of E_1 , E_3 and E_4 separately can be O and E_2 can be NH. In some embodiments, R_1 can be alkyl optionally substituted with a boronic ester or boronic ester. Typical boronic ester groups include, but are in no way limited to, $B(OMethyl)_2$, $B(OEthyl)_2$, $B(OPropyl)_2$, $B(OPhenyl)_2$, and the like.

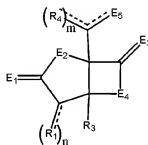
[0147] In a typical embodiment the compound is Salinosporamide A. In certain embodiments the HDACi can be selected from the group consisting of (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275), APHA compound 8, Apicidin, (-)-Depudecin, sodium butyrate, scriptaid, sirtinol, trichostatin A, valproic acid, vorinostat and the like. For example, the HDACi can be (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275), HDACi is valproic acid, vorinostat, and the like.

[0148] Some embodiments provide a method of inducing apoptosis of a cancer cell comprising contacting the cell with a HDACi and a compound having the structure of any one of Formulas I and II:



Formula I

and



Formula II

[0149] wherein:

[0150] the dashed lines represent a single or a double bond;

[0151] each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0152] n is 1 or 2, where if n is 2, then each R_1 can be the same or different;

[0153] m is 1 or 2, where if m is 2, then each R_4 can be the same or different;

[0154] R_2 is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 -

C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0155] R₃ is a halogen or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

[0156] each of E₁, E₃, E₄ and E₈ is an optionally substituted heteroatom;

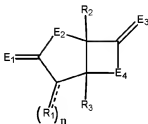
[0157] E₂ is an optionally substituted heteroatom or -CH₂- group; and

[0158] each R₄ is separately a halogen, a cyano, a nitro, an azido, or a thiocyno, or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

[0159] In some embodiments E₈ can be, for example, OH, O, OR₁₀, S, SR₁₁, SO₂R₁₁, NH, NH₂, NOH, NHOH, NR₁₂, and NHOR₁₃, wherein R₁₀₋₁₃ may separately include, for example, hydrogen, a substituted or unsubstituted of any of the following: alkyl, an aryl, a heteroaryl, and the like. R₃ can be methyl. Furthermore, R₄ may include a cyclohexyl. Also, each of E₁, E₃ and E₄ can be O and E₂ can be NH. Preferably, R₁ can be CH₂CH₂X, wherein X is selected from the group consisting of hydrogen, fluorine, chlorine, bromine, and iodine; wherein R₄ may include a cyclohexyl; wherein R₃ can be methyl; and wherein each of E₁, E₃ and E₄ separately can be O and E₂ can be NH. In some embodiments, R₁ can be alkyl optionally substituted with a boronic ester or boronic ester. For example, the boronic ester can be B(OMethyl)₂, B(OEthyl)₂, B(OPropyl)₂, B(OPhenyl)₂, and the like

[0160] In a typical embodiment the compound is Salinosporamide A. In certain embodiments the HDACi can be selected from the group consisting of (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275), APHA compound 8, Apicidin, (-)-Depudecin, sodium butyrate, scriptaid, sirtinol, trichostatin A, valproic acid, vorinostat and the like. For example, the HDACi can be (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275), HDACi is valproic acid, vorinostat, and the like.

[0161] Some embodiments provide a compound having the structure of Formula I:



Formula I

[0162] wherein:

[0163] the dashed lines represent a single or a double bond;

[0164] each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0165] n is 1 or 2, where if n is 2, then each R_1 can be the same or different;

[0166] R_2 is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxycarbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl,

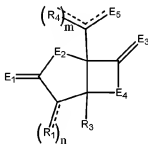
alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0167] R_3 is a halogen or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

[0168] each of E_1 , E_3 , and E_4 is an optionally substituted heteroatom; and

[0169] E_2 is an optionally substituted heteroatom or $-CH_2-$ group.

[0170] In some embodiments, compounds having the structure of Formula I have the structure of Formula II:



Formula II

[0171] wherein:

[0172] the dashed lines represent a single or a double bond;

[0173] each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

[0174] n is 1 or 2, where if n is 2, then each R_1 can be the same or different;

[0175] m is 1 or 2, where if m is 2, then each R_4 can be the same or different;

[0176] R_3 is a halogen or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

[0177] each of E_1 , E_3 , E_4 and E_5 is an optionally substituted heteroatom;

[0178] E_2 is an optionally substituted heteroatom or $-CH_2-$ group; and

[0179] each R_4 is separately a halogen, a cyano, a nitro, an azido, or a thiocyanato, or selected from the group consisting of optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

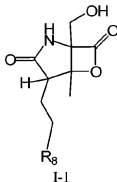
[0180] In some embodiments n can be equal to 1, while in others it can be equal to 2. When n is equal to 2, the substituents can be the same or can be different. Furthermore, in some embodiments R_3 is not a hydrogen. In some embodiments m can be equal to 1 or 2, and when m is equal to 2, R_4 can be the same or different.

[0181] In some embodiments E_5 can be, for example, OH, O, OR_{10} , S, SR_{11} , SO_2R_{11} , NH, NH_2 , NOH, $NHOH$, NR_{12} , and $NHOR_{13}$, wherein R_{10-13} may separately include, for example, hydrogen, a substituted or unsubstituted of any of the following: alkyl, an aryl, a heteroaryl, and the like. R_3 can be methyl. Furthermore, R_4 may include a cyclohexyl. Also, each of E_1 , E_3 and E_4 can be O and E_2 can be NH. Preferably, R_1 can be CH_2CH_2X , wherein X is selected from the group consisting of hydrogen, fluorine, chlorine, bromine, and iodine; wherein R_4 may include a cyclohexyl; wherein R_3 can be methyl; and wherein each of E_1 , E_3 and E_4 separately can be O and E_2 can be NH. In some embodiments, R_1 can be alkyl optionally substituted with a boronic ester or boronic ester. For example, the boronic ester can be $B(OMethyl)_2$, $B(OEthyl)_2$, $B(OPropyl)_2$, $B(OPhenyl)_2$, and the like

[0182] In some embodiments, **R₂** is not cyclohex-2-enyl carbinol when one of the **R₁** substituents is ethyl or chloroethyl and **R₃** is methyl.

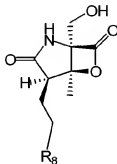
[0183] In some embodiments, **R₁** can be an optionally substituted C₁ to C₅ alkyl. For example, **R₁** can be methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, pentyl and the like. In some embodiments, **R₁** is not a substituted or unsubstituted, unbranched C₆ alkyl.

[0184] In a typical embodiment, **E₅** can be OH. For example, the compound may have the following Formula I-1:



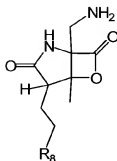
[0185] In some embodiments, for example, **R₈** can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

[0186] As an example, Formula I-1 may have the following stereochemistry:



[0187] In some embodiments, for example, **R₈** can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

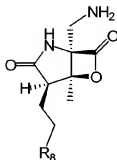
[0188] Still a further exemplary compound of Formula II is a compound having the following Formula I-2:



I-2

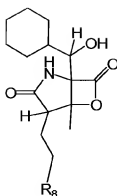
[0189] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

[0190] For example, Formula I-2 may have the following stereochemistry:



[0191] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

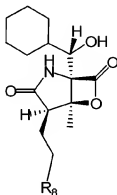
[0192] An exemplary compound of Formula II can have the following Formula II-1:



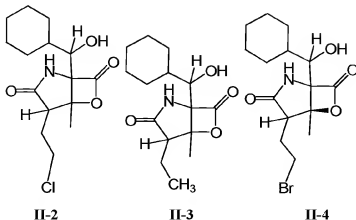
II-1

[0193] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

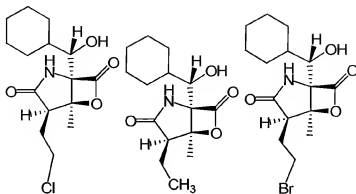
[0194] Exemplary stereochemistry can be as follows:



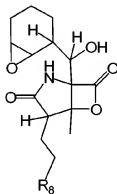
[0195] In some embodiments, the compound of Formula I can have any of the following structures of Formulae II-2, II-3, and II-4:



[0196] The following is exemplary stereochemistry for compounds having the structures of Fomulae II-2, II-3, and II-4, respectively:



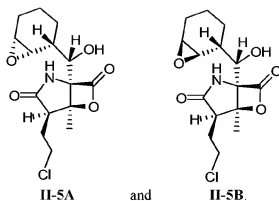
[0197] In other embodiments wherein R_4 may include a 7-oxa-bicyclo[4.1.0]hept-2-yl). An exemplary compound of Formula I is the following Formula II-5:



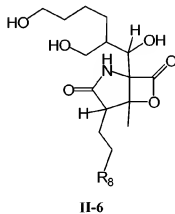
II-5.

[0198] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

[0199] The following are examples of compounds of Formula II-5 having the structures of Formulae II-5A and II-5B:

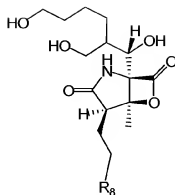


[0200] In still further embodiments, at least one R_4 may include an optionally substituted branched alkyl. For example, a compound of Formula I can be the following Formula II-6:

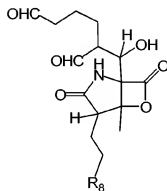


[0201] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

[0202] The following is exemplary stereochemistry for a compound of Formula II-6:



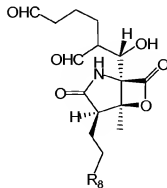
[0203] As another example, the compound of Formula I can be the following Formula II-7:



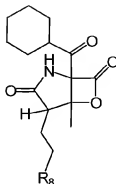
II-7

[0204] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

[0205] The following is exemplary stereochemistry for a compound having the structure of Formula II-7.



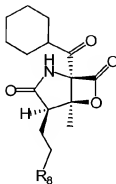
[0206] In other embodiments, at least one R_4 can be an optionally substituted cycloalkyl and E_5 can be an oxygen. For example, R_4 can be cyclopropyl, cyclopentyl, cyclohexyl, cycloheptyl, and the like. An exemplary compound of Formula I can have the structure of Formula II-8:



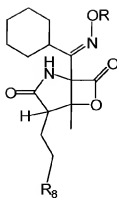
II-8

[0207] In some embodiments, R_8 can be, for example, hydrogen (II-8A), fluorine (II-8B), chlorine (II-8C), bromine (II-8D) and iodine (II-8E).

[0208] The following is exemplary stereochemistry for a compound having the structure of Formula II-8:



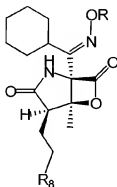
[0209] In some embodiments E_5 can be an amine oxide, giving rise to an oxime. An exemplary compound of Formula I has the following structure of Formula II-9:



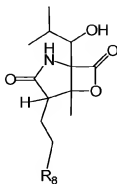
II-9

[0210] R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine; R can be a hydrogen, or an optionally substituted substituent selected from the group consisting of alkyl, aryl, heteroaryl, and the like.

[0211] The following is exemplary stereochemistry for a compound having the structure of Formula II-9:



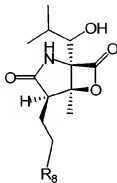
[0212] A further exemplary compound of Formula I has the following structure of Formula II-10:



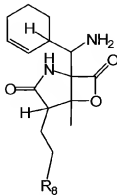
II-10

[0213] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

[0214] The following is exemplary stereochemistry for a compound having the structure of Formula II-10:



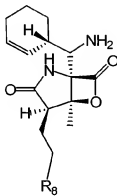
[0215] In some embodiments, E₅ can be NH₂. An exemplary compound of Formula I has the following structure of Formula II-11:



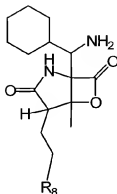
II-11

[0216] In some embodiments, for example, R₈ can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

[0217] The following is exemplary stereochemistry for a compound having the structure of Formula II-11:



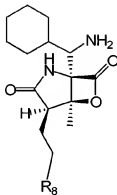
[0218] In some embodiments, at least one R_4 can be an optionally substituted cycloalkyl and E_5 can be NH_2 . For example, R_4 can be cyclopropyl, cyclopentyl, cyclohexyl, cycloheptyl, and the like. An exemplary compound of Formula I has the following structure of Formula II-12:



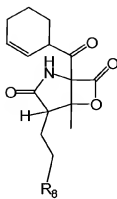
II-12

[0219] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

[0220] The following is exemplary stereochemistry for a compound having the structure of Formula II-12:

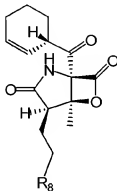


[0221] A further exemplary compound of Formula I has the following structure of Formula II-13:

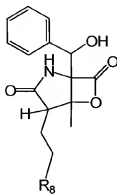


[0222] R_8 may include, for example, hydrogen (**II-13A**), fluorine (**II-13B**), chlorine (**II-13C**), bromine (**II-13D**) and iodine (**II-13E**).

[0223] The following is exemplary stereochemistry for a compound having the structure of Formula II-13:



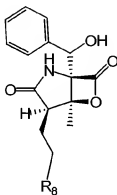
[0224] In another embodiment a compound of Formula I can have the following structure of Formula II-14:



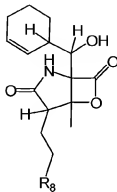
II-14

[0225] For example, R₈ can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine.

[0226] The following is exemplary stereochemistry for a compound having the structure of Formula II-14:



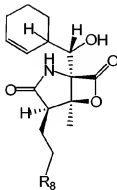
[0227] In another embodiment, for example, the radical R_4 of a compound of Formula II can be an optionally substituted cycloalkene. Furthermore, in some embodiments, the compounds of Formula II may include a hydroxy at E_8 , for example. A further exemplary compound of Formula II has the following structure of Formula II-15:



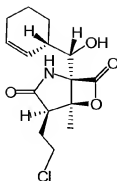
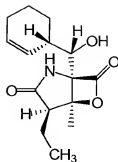
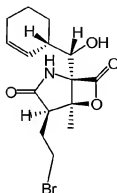
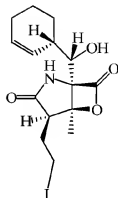
II-15

[0228] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine. R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0229] Exemplary stereochemistry can be as follows:

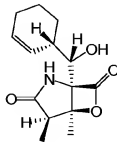


[0230] The following is exemplary stereochemistry for compounds having the structure of Formulae II-16, II-17, II-18, and II-19, respectively:

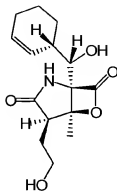
**II-16****II-17****II-18****II-19**

[0231] The compounds of Formulae II-16, II-17, II-18 and II-19 can be obtained by fermentation, synthesis, or semi-synthesis and isolated/purified as set forth below. Furthermore, the compounds of Formulae II-16, II-17, II-18 and II-19 can be used, and are referred to, as “starting materials” to make other compounds described herein.

[0232] In some embodiments, the compounds of Formula I, may include a methyl group as R₁, for example. A further exemplary compound, structure II-20, has the following structure and stereochemistry:

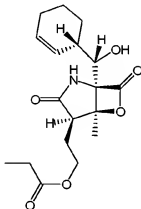
**II-20**

[0233] In some embodiments, the compounds of Formula I, may include hydroxyethyl as R_1 , for example. A further exemplary compound, Formula II-21, has the following structure and stereochemistry:



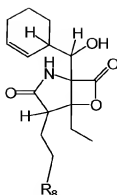
II-21

[0234] In some embodiments, the hydroxyl group of Formula II-21 can be esterified such that R_1 may include ethylpropionate, for example. An exemplary compound, structure II-22, has the following structure and stereochemistry:

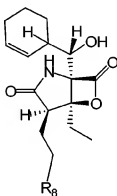


II-22

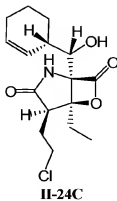
[0235] In some embodiments, the compounds of Formula I may include an ethyl group as R_3 , for example. A further exemplary compound of Formula I has the following structure of Formula II-23:

**II-23**

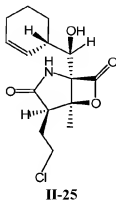
[0236] For example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine. Exemplary stereochemistry can be as follows:



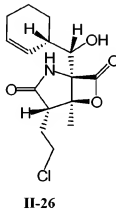
[0237] In some embodiments, the compounds of Formula II-23 may have the following structure and stereochemistry, exemplified by structure of Formula II-24C, where R_8 is chlorine:

**II-24C**

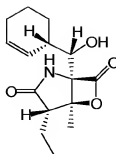
[0238] In some embodiments, the compounds of Formula II-15 may have the following stereochemistry, exemplified by the compound of Formula II-25, where R_8 is chlorine:



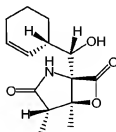
[0239] In some embodiments, the compound of Formula II-15 may have the following stereochemistry, exemplified by the compound of Formula II-26, where R_8 is chlorine:



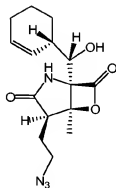
[0240] In some embodiments, the compound of Formula I may have the following structure and stereochemistry, exemplified by the structure of Formula II-27, where R_1 is ethyl:

**II-27**

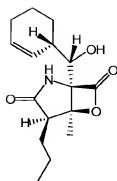
[0241] In some embodiments, the compound of Formula I may have the following structure and stereochemistry, exemplified by the structure of Formula II-28, where R_1 is methyl:

**II-28**

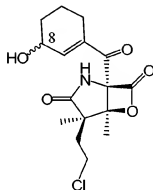
[0242] In some embodiments, the compounds of Formula I may include azidoethyl as R_1 , for example. A further exemplary compound, Formula II-29, has the following structure and stereochemistry:

**II-29**

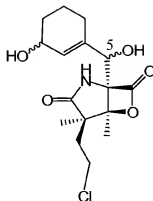
[0243] In some embodiments, the compounds of Formula I may include propyl as R_1 , for example. A further exemplary compound, Formula II-30, has the following structure and stereochemistry:

**II-30**

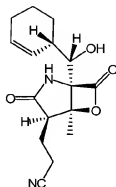
[0244] Still further exemplary compounds, Formulae II-31 and II-32, have the following structure and stereochemistry:

**II-31 and II-32**

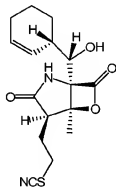
[0245] Other exemplary compounds, Formulae II-33, II-34, II-35 and II-36, have the following structure and stereochemistry:

**II-33 - II-36**

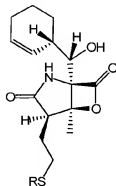
[0246] In some embodiments, the compound of Formula I may include cyanoethyl as R_1 ; for example, the compound of Formula II-37 has the following structure and stereochemistry:

**II-37**

[0247] In another embodiment, the compound of Formula I may include ethylthiocyanate as R_1 ; for example, the compound of Formula II-38 has the following structure and stereochemistry:

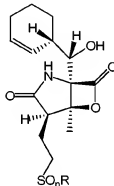
**II-38**

[0248] In some embodiments, the compounds of Formula I may include a thiol as R_1 , for example. A further exemplary compound, Formula II-39, has the following structure and stereochemistry, where $R = H$, alkyl, aryl, or substituted alkyl or aryl:



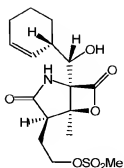
II-39

[0249] In a further exemplary compound, the sulfur of the compound of Formula II-39 can be oxidized to a sulfoxide ($n=1$) or sulfone ($n=2$), for example, as in the compound of structure II-40:

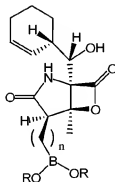


II-40

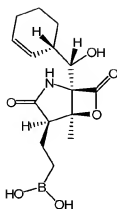
[0250] In some embodiments, the substituent R_1 of the compound of Formula I may include a leaving group, for example, a halogen, as in compounds of Formulae II-18 or II-19, or another leaving group, such as a sulfonate ester. One example is the methane sulfonate (mesylate) of Formula II-41:

**II-41**

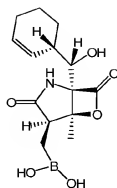
[0251] In some embodiments, the substituent R_1 of the compound of Formula I may include electron acceptors. The electron acceptor can be, for example, a Lewis acid, such as a boronic acid or ester. An exemplary compound, Formula II-42, has the following structure and stereochemistry, where $n = 0, 1, 2, 3, 4, 5$, or 6 , for example, and where $R = H$ or alkyl, for example:

**II-42**

[0252] Further exemplary compounds of Formula II-42 are the compounds of Formula II-42A, where $n=2$ and $R=H$, and the compound of Formula II-42B, where $n=1$ and $R=H$:

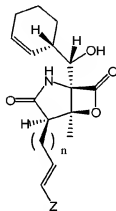


II-42A



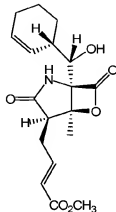
II-42B

[0253] In some embodiments where the substituent R_1 of the compound of Formula I includes an electron acceptor, the electron acceptor can be, for example, a Michael acceptor. An exemplary compound, structure II-43 has the following structure, where $n = 0, 1, 2, 3, 4, 5, 6$, and where Z is an electron withdrawing group, for example, CHO, COR, COOR, CONH₂, CN, NO₂, SOR, SO₂R, etc:



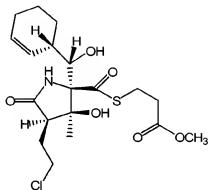
II-43

[0254] A further exemplary compound of Formula II-43 is the compound of structure II-43A, where $n=1$ and $Z=\text{CO}_2\text{CH}_3$:



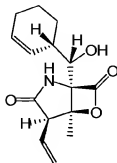
II-43A

[0255] In some embodiments, the compounds can be prodrug esters or thioesters of the compounds of Formula I. For example, the compound of Formula II-44 (a prodrug thioester of the compound of structure II-16) has the following structure and stereochemistry:



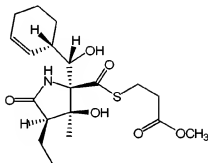
II-44

[0256] In some embodiments, the compounds of Formula I may include an alkenyl group as R_1 , for example, ethylenyl. A further exemplary compound, Formula II-46, has the following structure and stereochemistry:



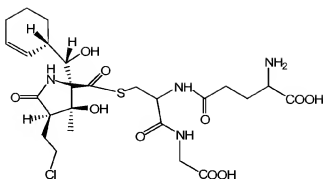
II-46

[0257] In some embodiments, the compounds can be prodrug esters or thioesters of the compounds of Formula I. For example, the compound of Formula II-47 (a prodrug thioester of the compound of structure II-17) has the following structure and stereochemistry:

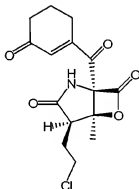


II-47

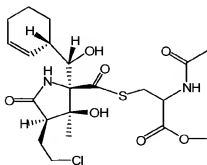
[0258] In some embodiments, the compounds can be prodrug esters or thioesters of the compounds of Formula I. For example, the compound of Formula II-48 has the following structure and stereochemistry:

**II-48**

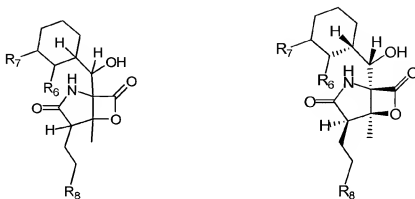
[0259] Another exemplary compound, structure II-49 has the following structure and stereochemistry:

**II-49**

[0260] In some embodiments, the compound can be prodrug ester or thioester of the compounds of Formula I. For example, the compound of Formula II-50 (prodrug ester of the compound of Formula II-16) has the following structure and stereochemistry:

**II-50**

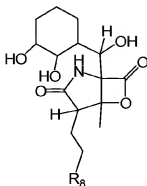
[0261] An exemplary compound of Formula I is the following Formula III-1, with and without exemplary stereochemistry:



III-1

[0262] In some embodiments, for example, R_8 can be selected from the group consisting of hydrogen, fluorine, chlorine, bromine and iodine. The substituent(s) R_6 and R_7 may each separately be selected from a hydrogen, a halogen, a nitro, a cyano, or an optionally substituted substituent selected from the group consisting of C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxy, carbonyl, alkoxy carbonyl, amino, aminocarbonyl, aminocarbonyloxy, azido, phenyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, and halogenated alkyl including polyhalogenated alkyl. Further, R_6 and R_7 both can be the same or different.

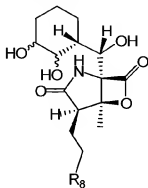
[0263] For example, an exemplary compound of Formula I has the following Formula III-2:



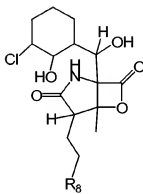
III-2

[0264] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0265] Exemplary stereochemistry can be as follows:



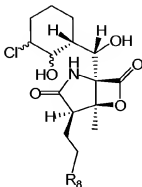
[0266] For example, an exemplary compound of Formula I has the following Formula III-3:



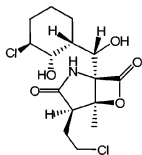
III-3

[0267] R_8 may include, for example, hydrogen (**III-3A**), fluorine (**III-3B**), chlorine (**III-3C**), bromine (**III-3D**) and iodine (**III-3E**).

[0268] Exemplary structure and stereochemistry can be as follows:

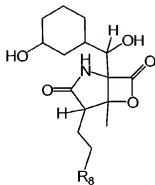


[0269] Additional exemplary structure and stereochemistry can be as follows:



III-3C

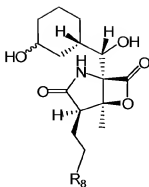
[0270] For example, an exemplary compound of Formula I has the following Formula III-4:



III-4

[0271] R_8 may include, for example, hydrogen, fluorine, chlorine, bromine and iodine.

[0272] Exemplary stereochemistry can be as follows:



[0273] Certain embodiments also provide pharmaceutically acceptable salts and pro-drug esters or thioesters of the compound of Formulae I and II, and provide methods of obtaining and purifying such compounds by the methods disclosed herein.

[0274] The term “pro-drug,” especially when referring to a pro-drug ester of the compound of Formula I synthesized by the methods disclosed herein, refers to a chemical derivative of the compound that is rapidly transformed *in vivo* to yield the compound, for example, by hydrolysis in blood or inside tissues. The term “pro-drug ester” refers to derivatives of the compounds disclosed herein formed by the addition of any of several ester- or thioester-forming groups that are hydrolyzed under physiological conditions. Examples of pro-drug ester groups include pivoyloxymethyl, acetoxymethyl, phthalidyl, indanyl and methoxymethyl, as well as other such groups known in the art, including a (5-*R*-2-oxo-1,3-dioxolen-4-yl)methyl group. Other prodrugs can be prepared by preparing a corresponding thioester of the compound, for example, by reacting with an appropriate thiol, such as thiophenol, Cysteine or derivatives thereof, or propanethiol, for example. Other examples of pro-drug ester groups can be found in, for example, T. Higuchi and V. Stella, in “Pro-drugs as Novel Delivery Systems”, Vol. 14, A.C.S. Symposium Series, American Chemical Society (1975); and “Bioreversible Carriers in Drug Design: Theory and Application”, edited by E. B. Roche, Pergamon Press: New York, 14-21 (1987) (providing examples of esters useful as prodrugs for compounds containing carboxyl groups). Each of the above-mentioned references is hereby incorporated by reference in its entirety.

[0275] The term “pharmaceutically acceptable salt,” as used herein, and particularly when referring to a pharmaceutically acceptable salt of a compound, including a compound of Formula I, as produced and synthesized by the methods disclosed herein, refers

to any pharmaceutically acceptable salts of a compound, and preferably refers to an acid addition salt of a compound. Preferred examples of pharmaceutically acceptable salt are the alkali metal salts (sodium or potassium), the alkaline earth metal salts (calcium or magnesium), or ammonium salts derived from ammonia or from pharmaceutically acceptable organic amines, for example C₁-C₇ alkylamine, cyclohexylamine, triethanolamine, ethylenediamine or tris-(hydroxymethyl)-aminomethane. With respect to compounds synthesized by the method of this embodiment that are basic amines, the preferred examples of pharmaceutically acceptable salts are acid addition salts of pharmaceutically acceptable inorganic or organic acids, for example, hydrohalic, sulfuric, phosphoric acid or aliphatic or aromatic carboxylic or sulfonic acid, for example acetic, succinic, lactic, malic, tartaric, citric, ascorbic, nicotinic, methanesulfonic, p-toluenesulfonic or naphthalenesulfonic acid.

[0276] Preferred pharmaceutical compositions disclosed herein include pharmaceutically acceptable salts and pro-drugs of the compound of Formula I obtained and purified by the methods disclosed herein. Accordingly, if the manufacture of pharmaceutical formulations involves intimate mixing of the pharmaceutical excipients and the active ingredient in its salt form, then it is preferred to use pharmaceutical excipients which are non-basic, that is, either acidic or neutral excipients.

[0277] It will be also appreciated that the phrase "compounds and compositions comprising the compound," or any like phrase, is meant to encompass compounds in any suitable form for pharmaceutical delivery, as discussed in further detail herein. For example, in certain embodiments, the compounds or compositions comprising the same may include a pharmaceutically acceptable salt of the compound.

[0278] The term "halogen atom," as used herein, means any one of the radio-stable atoms of column 7 of the Periodic Table of the Elements, *i.e.*, fluorine, chlorine, bromine, or iodine.

[0279] The term "alkyl," as used herein, means any unbranched or branched, substituted or unsubstituted, fully saturated (no double or triple bonds) hydrocarbon group. The alkyl group may have 1 to 24 carbon atoms (whenever it appears herein, a numerical range such as "1 to 24" refers to each integer in the given range; *e.g.*, "1 to 24 carbon atoms" means that the alkyl group may consist of 1 carbon atom, 2 carbon atoms, 3 carbon atoms,

etc., up to and including 24 carbon atoms, although the present definition also covers the occurrence of the term “alkyl” where no numerical range is designated). The alkyl group may also be a medium size alkyl having 1 to 10 carbon atoms. The alkyl group could also be a lower alkyl having 1 to 5 carbon atoms. The alkyl group of the compounds may be designated as “C₁₋₆ alkyl” or similar designations. By way of example only, “C₁₋₆ alkyl” indicates that there are one to six carbon atoms in the alkyl chain, i.e., the alkyl chain is selected from the group consisting of methyl, ethyl, propyl, iso-propyl, n-butyl, iso-butyl, sec-butyl, t-butyl, pentyl and hexyl. Typical alkyl groups include, but are in no way limited to, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, hexyl, and the like.

[0280] The term “substituted” has its ordinary meaning, as found in numerous contemporary patents from the related art. See, for example, U.S. Patent Nos. 6,509,331; 6,506,787; 6,500,825; 5,922,683; 5,886,210; 5,874,443; and 6,350,759; all of which are incorporated herein in their entireties by reference. Specifically, the definition of substituted is as broad as that provided in U.S. Patent No. 6,509,331, which defines the term “substituted alkyl” such that it refers to an alkyl group, preferably of from 1 to 10 carbon atoms, having from 1 to 5 substituents, and preferably 1 to 3 substituents, selected from the group consisting of alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, acyl, acylamino, acyloxy, amino, substituted amino, aminoacyl, aminoacyloxy, oxyacylamino, cyano, halogen, hydroxyl, carboxyl, carboxylalkyl, keto, thioketo, thiol, thioalkoxy, substituted thioalkoxy, thiocyanate, aryl, aryloxy, heteroaryl, heteroaryloxy, heterocyclic, heterocycloxy, hydroxyamino, alkoxyamino, nitro, azido, boronic acid, boronic ester, --SO-alkyl, --SO-substituted alkyl, --SO-aryl, --SO-heteroaryl, --SO₂-alkyl, --SO₂-substituted alkyl, --SO₂-aryl, --SO₂-heteroaryl, --OSO-alkyl, --OSO-substituted alkyl, --OSO-aryl, --OSO-heteroaryl, --OSO₂-alkyl, --OSO₂-substituted alkyl, --OSO₂-aryl, and --OSO₂-heteroaryl. The other above-listed patents also provide standard definitions for the term “substituted” that are well-understood by those of skill in the art.

[0281] The term “cycloalkyl” as used herein, refers to any non-aromatic hydrocarbon ring, preferably having three to twelve atoms comprising the ring.

[0282] The term “acyl” as used herein, refers to alkyl or aryl groups derived from an oxoacid, with an acetyl group being preferred.

[0283] The term “alkoxycarbonylacyl” as used herein, refers to an acyl group substituted with an alkoxycarbonyl group. Typical alkoxycarbonylacyl groups include, but are in no way limited to, $\text{CH}_3\text{OC(O)CH}_2\text{C(O)-}$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{OC(O)CH}_2\text{C(O)-}$, 4-ethoxycarbonylbenzoyl-, 4-methoxycarbonylbenzoyl-, 4-propoxycarbonylbenzoyl-, 3-tert-butoxycarbonylbenzoyl-, and the like.

[0284] The term “amino” as used herein, refers to amine radicals, wherein one or both hydrogen atoms are optionally replaced by substituents such as alkyl, and aryl groups. Typical amino groups include, but are in no way limited to, $-\text{NH}_2$, $-\text{NHMe}$, $-\text{NHEt}$, $-\text{NHCH}_2\text{phenyl}$, $-\text{N(Me)(phenyl)}$, $-\text{N(Et)(Me)}$, $-\text{N(Phenyl)(Et)}$, $-\text{N(Et)(CH}_2\text{phenyl)}$, $-\text{N(CH}_2\text{phenyl)(phenyl)}$, and the like.

[0285] The term “aminocarbonyl” and as used herein, refers to a carbonyl substituted with an amino. Typical aminocarbonyl groups include, but are in no way limited to, $-\text{C(O)NH}_2$, $-\text{C(O)NHMe}$, $-\text{C(O)NHEt}$, $-\text{C(O)NHCH}_2\text{phenyl}$, $-\text{C(O)N(Me)(phenyl)}$, $-\text{C(O)N(Et)(Me)}$, $-\text{C(O)N(Phenyl)(Et)}$, $-\text{C(O)N(Et)(CH}_2\text{phenyl)}$, $-\text{C(O)N(CH}_2\text{phenyl)(phenyl)}$, and the like.

[0286] The term “acyloxy” as used herein, refers to an acyl group attached to an oxygen with the oxygen being the attachment point. Typical acyloxy groups include, but are in no way limited to, MeC(O)O- , PhenylC(O)O- , and the like.

[0287] The term “alkenyl” as used herein, means any unbranched or branched, substituted or unsubstituted, unsaturated hydrocarbon including polyunsaturated hydrocarbons, with $\text{C}_1\text{-C}_6$ unbranched, mono-unsaturated and di-unsaturated, unsubstituted hydrocarbons being preferred, and mono-unsaturated, di-halogen substituted hydrocarbons being most preferred.

[0288] The term “cycloalkenyl” as used herein, refers to any non-aromatic hydrocarbon ring, preferably having five to twelve atoms comprising the ring and having at least one unsaturated bond.

[0289] The term “heterocycle” or “heterocyclic” refer to any non-aromatic cyclic compound containing one or more heteroatoms. In polycyclic ring systems, the one or more heteroatoms, may be present in only one of the rings. A heterocycle or heterocyclic group

may be substituted or unsubstituted. The substituted heterocycle or heterocyclic group can be substituted with any substituent, including those described above and those known in the art.

[0290] The term “aryl” as used herein, refers to a carbocyclic (all carbon) ring or two or more fused rings (rings that share two adjacent carbon atoms) that have a fully delocalized pi-electron system. Typical aryl groups include, but are in no way limited to, benzene, naphthalene, azulene and the like. An aryl group may be substituted or unsubstituted. The substituted aryls can be substituted with any substituent, including those described above and those known in the art.

[0291] The term “heteroaryl” as used herein, refers to an aromatic heterocyclic group, whether one ring or multiple fused rings. In fused ring systems, the one or more heteroatoms, may be present in only one of the rings. The hetero atom is an element other than carbon, including but not limited to, nitrogen, oxygen and sulfur. Typical heteroaryl groups include, but are in no way limited to, indole, oxazole, benzoxazole, isoxazole, benzisoxazole, thiazole, benzothiazole, isothiazole, imidazole, benzimidazole, pyrazole, pyridazine, pyridine, pyrimidine, purine, pyrazine, pteridine, pyrrole, phenoxazole, oxazole, isoxazole, oxadiazole, benzopyrazole, indazole, quinolizine, cinnoline, phthalazine, quinazoline, quinoxaline, and the like. A heteroaryl group of this invention may be substituted or unsubstituted. The substituted heteroaryls can be substituted with any substituent, including those described above and those known in the art.

[0292] The term “alkoxy” as used herein, refers to any unbranched, or branched, substituted or unsubstituted, saturated or unsaturated ether, with C₁-C₆ unbranched, saturated, unsubstituted ethers being preferred, with methoxy being preferred, and also with dimethyl, diethyl, methyl-isobutyl, and methyl-tert-butyl ethers also being preferred.

[0293] The term “cycloalkoxy” as used herein, refers to any cycloalkyl attached to an oxygen atom with the oxygen being the attachment point to the rest of the molecule.

[0294] The term “aryloalkoxy” as used herein, refers to an alkoxy group substituted with an aryl group. For example, aryloalkoxy can be methoxy substituted with an aryl group, such as benzyloxy and the like.

[0295] The term “aryloalkoxycarbonyl” as used herein, refers to an aryloalkoxy group attached to a carbonyl group with the carbonyl being the attachment point to the rest of

the molecule. Typical arylalkoxycarbonyl groups include, but are in no way limited to, benzyloxycarbonyl (i.e., $\text{PhenylCH}_2\text{OC(O)-}$) and the like.

[0296] The term “cycloalkyl” as used herein, refers to any non-aromatic hydrocarbon ring.

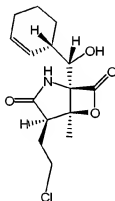
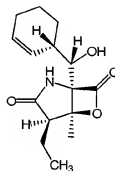
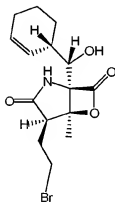
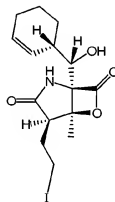
[0297] The term “alkoxycarbonyl” as used herein, refers to any linear, branched, cyclic, saturated, unsaturated, aliphatic or aryl alkoxy attached to a carbonyl group with the carbonyl group being the attachment point to the rest of the molecule. Typical alkoxycarbonyl groups include, but are in no way limited to, ethoxycarbonyl group, propyloxycarbonyl group, isopropyloxycarbonyl group, butoxycarbonyl group, sec-butoxycarbonyl group, tert-butoxycarbonyl group, cyclopentyloxycarbonyl group, cyclohexyloxycarbonyl group, benzyloxycarbonyl group, allyloxycarbonyl group, phenyloxycarbonyl group, pyridyloxycarbonyl group, and the like.

[0298] The term “alkoxycarbonyloxy” as used herein, refers to an alkoxycarbonyl group attached to an oxygen with the oxygen being the attachment point to the rest of the molecule. Typical alkoxycarbonyloxy groups include, but are in no way limited to, MeOC(O)O- , methoxycarbonyloxy group, ethoxycarbonyloxy group, propyloxycarbonyloxy group, isopropyloxycarbonyloxy group, butoxycarbonyloxy group, sec-butoxycarbonyloxy group, tert-butoxycarbonyloxy group, cyclopentyloxycarbonyloxy group, cyclohexyloxycarbonyloxy group, allyloxycarbonyloxy group, benzyloxycarbonyloxy group and the like. Additionally, alkoxycarbonyloxy groups refer to aryloxy and heteroaryloxy groups such as, phenyloxycarbonyloxy group, pyridyloxycarbonyloxy group, and the like.

[0299] The terms “pure,” “purified,” “substantially purified,” and “isolated” as used herein refer to the compound of the embodiment being free of other, dissimilar compounds with which the compound, if found in its natural state, would be associated in its natural state. In certain embodiments described as “pure,” “purified,” “substantially purified,” or “isolated” herein, the compound may comprise at least 0.5%, 1%, 5%, 10%, or 20%, and most preferably at least 50% or 75% of the mass, by weight, of a given sample.

[0300] The terms “derivative,” “variant,” or other similar term refers to a compound that is an analog of the other compound.

[0301] Certain of the compounds of Formula I can be obtained and purified or can be obtained via semi-synthesis from purified compounds as set forth herein. Generally, without being limited thereto, the compounds of Formula II-15, preferably, Formulae II-16 (Salinosporamide A), II-17, II-18 and II-19, can be obtained synthetically or by fermentation. Exemplary fermentation procedures are provided below. Further, the compounds of structure II-15, preferably, Formulae II-16, II-17, II-18 and II-19 can be used as starting compounds in order to obtain/synthesize various of the other compounds described herein. Exemplary non-limiting syntheses are provided herein.

**II-16****II-17****II-18****II-19**

[0302] The compound of Formula II-16 may be produced through a high-yield saline fermentation (~350 - 400 mg/L) and modifications of the conditions have yielded new analogs in the fermentation extracts. Additional analogs can be generated through directed biosynthesis. Directed biosynthesis is the modification of a natural product by adding biosynthetic precursor analogs to the fermentation of producing microorganisms (Lam, *et al.*,

J. Antibiot (Tokyo) 44:934 (1991), Lam, *et al.*, *J. Antibiot (Tokyo)* 54:1 (2001); which is hereby incorporated by reference in its entirety).

[0303] Exposing the producing culture to analogs of acetic acid, phenylalanine, valine, butyric acid, shikimic acid, and halogens, preferably, other than chlorine, can lead to the formation of new analogs. The new analogs produced can be easily detected in crude extracts by HPLC and LC-MS. For example, after manipulating the medium with different concentrations of sodium bromide, a bromo-analog, the compound of Formula II-18, was successfully produced in shake-flask culture at a titer of 14 mg/L.

[0304] A second approach to generate analogs is through biotransformation. Biotransformation reactions are chemical reactions catalyzed by enzymes or whole cells containing these enzymes. Zaks, A., *Curr Opin Chem Biol* 5:130 (2001). Microbial natural products are ideal substrates for biotransformation reactions as they are synthesized by a series of enzymatic reactions inside microbial cells. Riva, S., *Curr Opin Chem Biol* 5:106 (2001).

[0305] Given the structure of the described compounds, including those of Formula I-15, for example, the possible biosynthetic origins are acetyl-CoA, ethylmalonyl-CoA, phenylalanine and chlorine. Ethylmalonyl-CoA is derived from butyryl-CoA, which can be derived either from valine or crotonyl-CoA. Liu, *et al.*, *Metab Eng* 3:40 (2001). Phenylalanine is derived from shikimic acid.

[0306] Alternatively, compounds such as structure II-16 and its analogs may be produced synthetically, e.g., such as described in United States Application Serial No. 11/697,689, which is incorporated by reference in its entirety.

Production of Compounds of Formulae I-7, II-16, II-17, II-18, II-20, II-24C, II-26, II-27 and II-28

[0307] The production of compounds of Formulae I-7, II-16, II-17, II-18, II-20, II-24C, II-26, II-27 and II-28 can be carried out by cultivating strain CNB476 and strain NPS21184, a natural variant of strain CNB476, in a suitable nutrient medium under conditions described herein, preferably under submerged aerobic conditions, until a substantial amount of compounds are detected in the fermentation; harvesting by extracting

the active components from the fermentation broth with a suitable solvent; concentrating the solvent containing the desired components; then subjecting the concentrated material to chromatographic separation to isolate the compounds from other metabolites also present in the cultivation medium.

[0308] The culture (CNB476) was deposited on June 20, 2003 with the American Type Culture Collection (ATCC) in Rockville, MD and assigned the ATCC patent deposition number PTA-5275. Strain NPS21184, a natural variant of strain CNB476, was derived from strain CNB476 as a single colony isolate. Strain NPS21184 has been deposited to ATCC on April 27, 2005. The ATCC deposit meets all of the requirements of the Budapest treaty. The culture is also maintained at and available from Nereus Pharmaceutical Culture Collection at 10480 Wateridge Circle, San Diego, CA 92121. In addition to the specific microorganism described herein, it should be understood that mutants, such as those produced by the use of chemical or physical mutagens including X-rays, etc. and organisms whose genetic makeup has been modified by molecular biology techniques, may also be cultivated to produce the starting compounds of Formulae II-16, II-17, and II-18.

Fermentation of strain CNB476 and strain NPS21184

[0309] Production of compounds can be achieved at temperature conducive to satisfactory growth of the producing organism, e.g. from 16°C to 40°C, but it is preferable to conduct the fermentation at 22°C to 32°C. The aqueous medium can be incubated for a period of time necessary to complete the production of compounds as monitored by high pressure liquid chromatography (HPLC), preferably for a period of about 2 to 10 days, on a rotary shaker operating at about 50 rpm to 400 rpm, preferably at 150 rpm to 250 rpm, for example. The production of the compounds can also be achieved by cultivating the production strain in a bioreactor, such as a fermentor system that is suitable for the growth of the production strain.

[0310] Growth of the microorganisms can be achieved by one of ordinary skill of the art by the use of appropriate medium. Broadly, the sources of carbon include glucose, fructose, mannose, maltose, galactose, mannitol and glycerol, other sugars and sugar alcohols, starches and other carbohydrates, or carbohydrate derivatives such as dextran,

cerelose, as well as complex nutrients such as oat flour, corn meal, millet, corn, and the like. The exact quantity of the carbon source that is utilized in the medium will depend in part, upon the other ingredients in the medium, but an amount of carbohydrate between 0.5 to 25 percent by weight of the medium can be satisfactorily used, for example. These carbon sources can be used individually or several such carbon sources can be combined in the same medium, for example. Certain carbon sources are preferred as hereinafter set forth.

[0311] The sources of nitrogen include amino acids such as glycine, arginine, threonine, methionine and the like, ammonium salt, as well as complex sources such as yeast extracts, corn steep liquors, distiller solubles, soybean meal, cottonseed meal, fish meal, peptone, and the like. The various sources of nitrogen can be used alone or in combination in amounts ranging from 0.5 to 25 percent by weight of the medium, for example.

[0312] Among the nutrient inorganic salts, which can be incorporated in the culture media, are the customary salts capable of yielding sodium, potassium, magnesium, calcium, phosphate, sulfate, chloride, carbonate, and like ions. Also included are trace metals such as cobalt, manganese, iron, molybdenum, zinc, cadmium, and the like.

Pharmaceutical Compositions

[0313] In one embodiment, the compounds disclosed herein are used in pharmaceutical compositions. The compounds preferably can be produced by the methods disclosed herein. The compounds can be used, for example, in pharmaceutical compositions comprising a pharmaceutically acceptable carrier prepared for storage and subsequent administration. Also, embodiments relate to a pharmaceutically effective amount of the products and compounds disclosed above in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985), which is incorporated herein by reference in its entirety. Preservatives, stabilizers, dyes and even flavoring agents can be provided in the pharmaceutical composition. For example, sodium benzoate, ascorbic acid and esters of p-hydroxybenzoic acid can be added as preservatives. In addition, antioxidants and suspending agents can be used.

[0314] The compositions can be formulated and used as tablets, capsules, or elixirs for oral administration; suppositories for rectal administration; sterile solutions, suspensions for injectable administration; patches for transdermal administration, and subdermal deposits and the like. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (for example, liposomes), can be utilized.

[0315] Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or other organic oils such as soybean, grapefruit or almond oils, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[0316] Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Dragee cores are

provided with suitable coatings. For this purpose, concentrated sugar solutions can be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. For this purpose, concentrated sugar solutions can be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. Such formulations can be made using methods known in the art (see, for example, U.S. Patent Nos. 5,733,888 (injectable compositions); 5,726,181 (poorly water soluble compounds); 5,707,641 (therapeutically active proteins or peptides); 5,667,809 (lipophilic agents); 5,576,012 (solubilizing polymeric agents); 5,707,615 (anti-viral formulations); 5,683,676 (particulate medicaments); 5,654,286 (topical formulations); 5,688,529 (oral suspensions); 5,445,829 (extended release formulations); 5,653,987 (liquid formulations); 5,641,515 (controlled release formulations) and 5,601,845 (spheroid formulations); all of which are incorporated herein by reference in their entireties.

[0317] Further disclosed herein are various pharmaceutical compositions well known in the pharmaceutical art for uses that include topical, intraocular, intranasal, and intraauricular delivery. Pharmaceutical formulations include aqueous ophthalmic solutions of the active compounds in water-soluble form, such as eyedrops, or in gellan gum (Shedden et al., *Clin. Ther.*, 23(3):440-50 (2001)) or hydrogels (Mayer et al., *Ophthalmologica*, 210(2):101-3 (1996)); ophthalmic ointments; ophthalmic suspensions, such as microparticulates, drug-containing small polymeric particles that are suspended in a liquid carrier medium (Joshi, A. 1994 *J Ocul Pharmacol* 10:29-45), lipid-soluble formulations (Alm et al., *Prog. Clin. Biol. Res.*, 312:447-58 (1989)), and microspheres (Mordenti, *Toxicol. Sci.*, 52(1):101-6 (1999)); and ocular inserts. All of the above-mentioned references, are incorporated herein by reference in their entireties. Such suitable pharmaceutical formulations are most often and preferably formulated to be sterile, isotonic and buffered for stability and comfort. Pharmaceutical compositions may also include drops and sprays often

prepared to simulate in many respects nasal secretions to ensure maintenance of normal ciliary action. As disclosed in Remington's Pharmaceutical Sciences (Mack Publishing, 18th Edition), which is incorporated herein by reference in its entirety, and well-known to those skilled in the art, suitable formulations are most often and preferably isotonic, slightly buffered to maintain a pH of 5.5 to 6.5, and most often and preferably include anti-microbial preservatives and appropriate drug stabilizers. Pharmaceutical formulations for intraauricular delivery include suspensions and ointments for topical application in the ear. Common solvents for such aural formulations include glycerin and water.

[0318] To formulate the compounds of Formulae I and II as an anti-cancer agent, known surface active agents, excipients, smoothing agents, suspension agents and pharmaceutically acceptable film-forming substances and coating assistants, and the like can be used. Preferably alcohols, esters, sulfated aliphatic alcohols, and the like can be used as surface active agents; sucrose, glucose, lactose, starch, crystallized cellulose, mannitol, light anhydrous silicate, magnesium aluminate, magnesium methasilicate aluminate, synthetic aluminum silicate, calcium carbonate, sodium acid carbonate, calcium hydrogen phosphate, calcium carboxymethyl cellulose, and the like can be used as excipients; magnesium stearate, talc, hardened oil and the like can be used as smoothing agents; coconut oil, olive oil, sesame oil, peanut oil, soya can be used as suspension agents or lubricants; cellulose acetate phthalate as a derivative of a carbohydrate such as cellulose or sugar, or methylacetate-methacrylate copolymer as a derivative of polyvinyl can be used as suspension agents; and plasticizers such as ester phthalates and the like can be used as suspension agents. In addition to the foregoing preferred ingredients, sweeteners, fragrances, colorants, preservatives and the like can be added to the administered formulation of the compound produced by the method of the embodiment, particularly when the compound is to be administered orally.

[0319] When used as an anti-cancer compound, for example, the compounds of Formulae I and II or compositions including compounds of Formulae I and II can be administered by either oral or non-oral pathways. When administered orally, it can be administered in capsule, tablet, granule, spray, syrup, or other such form. When administered non-orally, it can be administered as an aqueous suspension, an oily preparation or the like or

as a drip, suppository, salve, ointment or the like, when administered via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, or the like.

[0320] In one embodiment, the anti-cancer agent can be mixed with additional substances to enhance their effectiveness.

Methods of Administration

[0321] In an alternative embodiment, the disclosed chemical compounds and the disclosed pharmaceutical compositions are administered by a particular method as an anti-cancer, anti-microbial or anti-inflammatory. Such methods include, among others, (a) administration through oral pathways, which administration includes administration in capsule, tablet, granule, spray, syrup, or other such forms; (b) administration through non-oral pathways, which administration includes administration as an aqueous suspension, an oily preparation or the like or as a drip, suppository, salve, ointment or the like; administration via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, intradermally, or the like; as well as (c) administration topically, (d) administration rectally, or (e) administration vaginally, as deemed appropriate by those of skill in the art for bringing the compound of the present embodiment into contact with living tissue; and (f) administration via controlled released formulations, depot formulations, and infusion pump delivery. As further examples of such modes of administration and as further disclosure of modes of administration, disclosed herein are various methods for administration of the disclosed chemical compounds and pharmaceutical compositions including modes of administration through intraocular, intranasal, and intraauricular pathways.

[0322] The pharmaceutically effective amount of the compositions that include the described compounds required as a dose will depend on the route of administration, the type of animal, including human, being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize. In a typical embodiment, a compound represented by Formulae I and II can be administered to a patient in need of an anti-cancer agent, until the need is effectively reduced or preferably removed.

[0323] In practicing the methods of the embodiment, the products or compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized *in vivo*, ordinarily in a mammal, preferably in a human, or *in vitro*. In employing them *in vivo*, the products or compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, vaginally, nasally or intraperitoneally, employing a variety of dosage forms. Such methods may also be applied to testing chemical activity *in vivo*.

[0324] As will be readily apparent to one skilled in the art, the useful *in vivo* dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular compounds employed, and the specific use for which these compounds are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, can be accomplished by one skilled in the art using routine pharmacological methods. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. Alternatively, acceptable *in vitro* studies can be used to establish useful doses and routes of administration of the compositions identified by the present methods using established pharmacological methods.

[0325] In non-human animal studies, applications of potential products are commenced at higher dosage levels, with dosage being decreased until the desired effect is no longer achieved or adverse side effects disappear. The dosage may range broadly, depending upon the desired affects and the therapeutic indication. Typically, dosages can be between about 10 mg/kg and 100 mg/kg body weight, preferably between about 100 mg/kg and 10 mg/kg body weight. Alternatively dosages can be based and calculated upon the surface area of the patient, as understood by those of skill in the art. Administration is preferably oral on a daily or twice daily basis.

[0326] The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. See for example, Fingl *et al.*, in *The Pharmacological Basis of Therapeutics*, 1975, which is incorporated herein by reference in its entirety. It should be noted that the attending physician would know how to and when

to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above can be used in veterinary medicine.

[0327] Depending on the specific conditions being treated, such agents can be formulated and administered systemically or locally. A variety of techniques for formulation and administration can be found in Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA (1990), which is incorporated herein by reference in its entirety. Suitable administration routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

[0328] For injection, the agents of the embodiment can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the embodiment into dosages suitable for systemic administration is within the scope of the embodiment. With proper choice of carrier and suitable manufacturing practice, the compositions disclosed herein, in particular, those formulated as solutions, can be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the embodiment to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

[0329] Agents intended to be administered intracellularly can be administered using techniques well known to those of ordinary skill in the art. For example, such agents can be encapsulated into liposomes, then administered as described above. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external micro-environment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules can be directly administered intracellularly.

[0330] Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration can be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions can be manufactured in a manner that is itself known, for example, by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

[0331] Compounds disclosed herein can be evaluated for efficacy and toxicity using known methods. For example, the toxicology of a particular compound, or of a subset of the compounds, sharing certain chemical moieties, can be established by determining *in vitro* toxicity towards a cell line, such as a mammalian, and preferably human, cell line. The results of such studies are often predictive of toxicity in animals, such as mammals, or more specifically, humans. Alternatively, the toxicity of particular compounds in an animal model, such as mice, rats, rabbits, dogs or monkeys, can be determined using known methods. The efficacy of a particular compound can be established using several art recognized methods, such as *in vitro* methods, animal models, or human clinical trials. Art-recognized *in vitro* models exist for nearly every class of condition, including the conditions abated by the compounds disclosed herein, including cancer, cardiovascular disease, and various immune dysfunction, and infectious diseases. Similarly, acceptable animal models can be used to establish efficacy of chemicals to treat such conditions. When selecting a model to determine

efficacy, the skilled artisan can be guided by the state of the art to choose an appropriate model, dose, and route of administration, and regime. Of course, human clinical trials can also be used to determine the efficacy of a compound in humans.

[0332] In the case of using a compound produced by methods of the embodiment as a biochemical test reagent, the compound produced by methods of the embodiment inhibits the progression of the disease when it is dissolved in an organic solvent or hydrous organic solvent and it is directly applied to any of various cultured cell systems. Usable organic solvents include, for example, methanol, methylsulfoxide, and the like. The formulation can, for example, be a powder, granular or other solid inhibitor, or a liquid inhibitor prepared using an organic solvent or a hydrous organic solvent. While a preferred concentration of the compound produced by the method of the embodiment for use as an anticancer compound is generally in the range of about 1 to about 100 $\mu\text{g/mL}$, the most appropriate use amount varies depending on the type of cultured cell system and the purpose of use, as will be appreciated by persons of ordinary skill in the art. Also, in certain applications it can be necessary or preferred to persons of ordinary skill in the art to use an amount outside the foregoing range.

[0333] As will be understood by one of skill in the art, "need" is not an absolute term and merely implies that the patient can benefit from the treatment of the anti-cancer agent in use. By "patient" what is meant is an organism that can benefit by the use of an anti-cancer agent. For example, any organism with cancer, such as, pancreatic cancer. In one embodiment, the patient's health may not require that an anti-cancer agent be administered, however, the patient may still obtain some benefit by the reduction of the level of cancer cells present in the patient, and thus be in need. In one embodiment, the anti- anti-cancer agent is effective against one type of cancer, but not against other types; thus, allowing a high degree of selectivity in the treatment of the patient. In choosing such an anti-cancer agent, the methods and results disclosed in the Examples can be useful. In still further embodiments, the anti-cancer agent is effective against a broad spectrum of cancers or all cancers. Examples of cancers, against which the compounds can be effective include pancreatic cancer, a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma, multiple myelomas, a melanoma, and the like.

[0334] “Therapeutically effective amount,” “pharmaceutically effective amount,” or similar term, means that amount of drug or pharmaceutical agent that will result in a biological or medical response of a cell, tissue, system, animal, or human that is being sought. In a preferred embodiment, the medical response is one sought by a researcher, veterinarian, medical doctor, or other clinician.

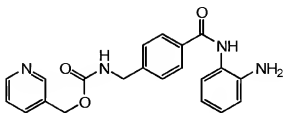
[0335] “Anti-cancer agent” refers to a compound or composition including the compound that reduces the likelihood of survival of a cancer cell. In one embodiment, the likelihood of survival is determined as a function of an individual cancer cell; thus, the anti-cancer agent will increase the chance that an individual cancer cell will die. In one embodiment, the likelihood of survival is determined as a function of a population of cancer cells; thus, the anti-cancer agent will increase the chances that there will be a decrease in the population of cancer cells. In one embodiment, anti-cancer agent means chemotherapeutic agent or other similar term.

[0336] A “chemotherapeutic agent” is a chemical compound useful in the treatment of a neoplastic disease, such as cancer. Examples of chemotherapeutic agents include alkylating agents, such as a nitrogen mustard, an ethyleneimine and a methylmelamine, an alkyl sulfonate, a nitrosourea, and a triazene, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, corticosteroids, a natural product such as a vinca alkaloid, an epipodophyllotoxin, an antibiotic, an enzyme, a taxane, and a biological response modifier or antibodies to biological response modifiers or other agents; miscellaneous agents such as a platinum coordination complex, an anthracenedione, an anthracycline, a substituted urea, a methyl hydrazine derivative, or an adrenocortical suppressant; or a hormone or an antagonist such as an adrenocorticosteroid, a progestin, an estrogen, an antiestrogen, an androgen, an antiandrogen, or a gonadotropin-releasing hormone analog. Specific examples include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside (“Ara-C”), Cyclophosphamide, Thiotepa, Busulfan, Cytosin, Taxol, Toxotere, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincristine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins,

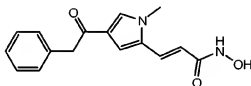
Esperamicins, Melphalan, and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

[0337] Additional examples of such chemotherapeutics include alkaloids, alkylating agents, antibiotics, antimetabolites, enzymes, hormones, platinum compounds, immunotherapeutics (antibodies, T-cells, epitopes), BRMs, and the like. Examples include, Vincristine, Vinblastine, Vindesine, Paclitaxel (Taxol), Docetaxel, topoisomerase inhibitors epipodophyllotoxins (Etoposide (VP-16), Camptothecin, nitrogen mustards (cyclophosphamide Cytoxan), Nitrosoureas, Camustine, lomustine, dacarbazine, hydroxymethylmelamine, thiotepa and mitocycin C, Dactinomycin (Actinomycin D), anthracycline antibiotics (Daunorubicin, Daunomycin, Cerubidine), Doxorubicin (Adriamycin), Idarubicin (Idamycin), Anthracenediones (Mitoxantrone), Bleomycin (Blenoxane), Plicamycin (Mithramycin, Antifolates (Methotrexate (Folex, Mexate)), purine antimetabolites (6-mercaptopurine (6-MP, Purinethol) and 6-thioguanine (6-TG). The two major anticancer drugs in this category are 6-mercaptopurine and 6-thioguanine, Chlorodeoxyadenosine and Pentostatin, Pentostatin (2'-deoxycofomycin), pyrimidine antagonists, Avastin, Leucovorin, Oxaliplatin, fluoropyrimidines (5-fluorouracil(Adrucil), 5-fluorodeoxyuridine (FdUrd) (Floxuridine)), Cytosine Arabinoside (Cytosar, ara-C), Fludarabine, L-asparaginase, Hydroxyurea, glucocorticoids, antiestrogens, tamoxifen, nonsteroidal antiandrogens, flutamide, aromatase inhibitors Anastrozole(Arimidex), Cisplatin, 6-Mercaptopurine and Thioguanine, Methotrexate, Cytoxan, Cytarabine, L-Asparaginase, Steroids: Prednisone and Dexamethasone, bevacizumab, and gemcitabine. Also, proteasome inhibitors such as bortezomib and carfilzomib (PR-171) can be used in combination with the instant compounds, for example. Examples of biologics can include agents such as TRAIL, antibodies to TRAIL and agonistic antibodies TRAIL death receptors DR4 and DR5, integrins such as alpha-V-beta-3 ($\alpha V\beta 3$) and / or other cytokine/growth factors that are involved in angiogenesis, VEGF, EGF, FGF and PDGF and antibodies to these cytokines/growth factors such as Erbitux. In some aspects, the compounds can be conjugated to or delivered with an antibody.

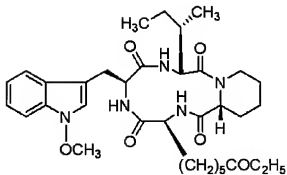
[0338] In some embodiments, the additional chemotherapeutic is a histone deacetylase inhibitor (HDACi). In various embodiments, the HDACi is selected from the group consisting of:



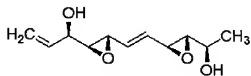
(pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate
(MS-275 or SNDX-275),



APHA compound 8,



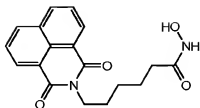
apicidin,



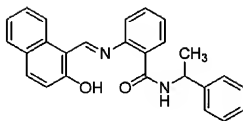
(-)-Depudecin,



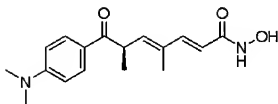
sodium Butyrate,



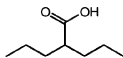
Scriptaid,



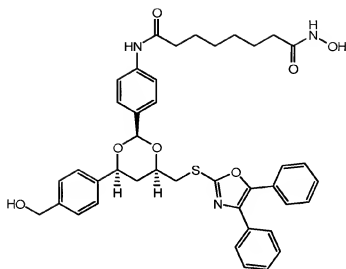
Sirtinol,



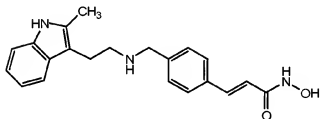
trichostatin A,



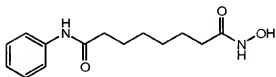
valproic acid,



tubacin,

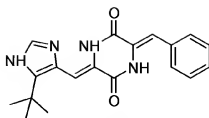


panobinostat, and



vorinostat (suberoylanilide hydroxamic acid (SAHA)).

[0339] In other embodiments, the additional chemotherapeutic is a vascular disrupting agents (VDA). Examples of such VDAs include combrostatatin CA4P and NPI-2358. NPI-2358 is represented by the following formula:

**NPI-2358**

[0340] The anti-cancer agent may act directly upon a cancer cell to kill the cell, induce death of the cell, to prevent division of the cell, and the like. Alternatively, the anti-cancer agent may indirectly act upon the cancer cell by limiting nutrient or blood supply to the cell, for example. Such anti-cancer agents are capable of destroying or suppressing the growth or reproduction of cancer cells, such as a colorectal carcinoma, a prostate carcinoma, a breast adenocarcinoma, a non-small cell lung carcinoma, an ovarian carcinoma, multiple myelomas, a melanoma, and the like.

[0341] In one embodiment, a described compound, preferably a compound having the Formula I, including those as described herein, is considered an effective anti-cancer agent if the compound can influence 10% of the cancer cells, for example. In a more preferred embodiment, the compound is effective if it can influence 10 to 50% of the cancer cells. In an even more preferred embodiment, the compound is effective if it can influence 50-80% of the cancer cells. In an even more preferred embodiment, the compound is effective if it can influence 80-95% of the cancer cells. In an even more preferred embodiment, the compound is effective if it can influence 95-99% of the cancer cells. "Influence" is defined by the mechanism of action for each compound. For example, if a compound prevents the division of cancer cells, then influence is a measure of prevention of cancer cell division. Not all mechanisms of action need be at the same percentage of effectiveness. In an alternative embodiment, a low percentage effectiveness can be desirable if the lower degree of effectiveness is offset by other factors, such as the specificity of the compound, for example. Thus a compound that is only 10% effective, for example, but displays little in the way of harmful side-effects to the host, or non-harmful microbes or cells, can still be considered effective.

[0342] In one embodiment, the compounds described herein are administered simply to remove cancer cells and need not be administered to a patient. For example, the compounds can be administered *ex vivo* to a cell sample, such as a bone marrow or stem cell transplant to ensure that only non-cancerous cells are introduced into the recipient. After the compounds are administered they may optionally be removed. Whether or not this is an option will depend upon the relative needs of the situation and the risks associated with the compound, which in part can be determined as described in the Examples below.

[0343] The following non-limiting examples are meant to describe the preferred embodiments of the methods. Variations in the details of the particular methods employed and in the precise chemical compositions obtained will undoubtedly be appreciated by those of skill in the art.

EXAMPLES

EXAMPLE 1

FERMENTATION OF COMPOUND OF FORMULAE I-7, II-16, II-17, II-20, II-24C, II-26 AND II-28 USING STRAIN CNB476

[0344] Strain CNB476 was grown in a 500-mL flask containing 100 mL of vegetative medium consisting of the following per liter of deionized water: glucose, 4 g; Bacto tryptone, 3 g; Bacto casitone, 5 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28°C for 3 days on a rotary shaker operating at 250 rpm. Five mL each of the first seed culture was inoculated into three 500-ml flasks containing of 100 mL of the vegetative medium. The second seed cultures were incubated at 28°C and 250 rpm on a rotary shaker for 2 days. Five mL each of the second seed culture was inoculated into thirty-five 500-mL flasks containing of 100 mL of the vegetative medium. The third seed cultures were incubated at 28°C and 250 rpm on a rotary shaker for 2 days. Five mL each of the third seed culture was inoculated into four hundred 500-mL flasks containing 100 mL of the Production Medium A consisting of the following per liter of deionized water: starch, 10 g; yeast extract, 4 g; Hy-Soy, 4 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The production cultures were incubated at 28°C and 250 rpm on

roarry shakers for 1 day. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were added to the production cultures. The production cultures were further incubated at 28°C and 250 rpm on rotary shakers for 5 days and achieved a titer of Compound II-16 of about 200 mg/L. The culture broth was filtered through cheese cloth to recover the Amberlite XAD-7 resin. The resin was extracted with 2 times 6 liters ethyl acetate followed by 1 time 1.5 liters ethyl acetate. The combined extracts were dried in vacuo. The dried extract, containing 3.8 grams the compound of Formula II-16 and lesser quantities of compounds of formulae II-20 and II-24C, was then processed for the recovery of the compounds of Formula I-7, II-16, II-20, II-24C, II-26 and II-28.

EXAMPLE 2

FERMENTATION OF COMPOUNDS I-7, II-16, II-17, II-20, II-24C, II-26 AND II-28 USING STRAIN NPS21184

[0345] Strain NPS21184 was grown in a 500-mL flask containing 100 mL of vegetative medium consisting of the following per liter of deionized water: glucose, 8 g; yeast extract, 6 g; Hy-Soy, 6 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28°C for 3 days on a rotary shaker operating at 250 rpm. Five mL of the first seed culture was inoculated into 500-mL flask containing of 100 mL of the vegetative medium. The second seed cultures were incubated at 28°C and 250 rpm on a rotary shaker for 2 days. Five mL each of the second seed culture was inoculated into 500-mL flask containing of 100 mL of the vegetative medium. The third seed cultures were incubated at 28°C and 250 rpm on a rotary shaker for 2 days. Five mL each of the third seed culture was inoculated into 500-mL flask containing 100 mL of the Production Medium B consisting of the following per liter of deionized water: starch, 20 g; yeast extract, 4 g; Hy-Soy, 8 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The production cultures were incubated at 28°C and 250 rpm on rotary shakers for 1 day. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were added to the production culture. The production culture was further incubated at 28°C and 250 rpm on rotary shaker for 4 days and achieved a titer of 350 – 400 mg/L for Compound II-16.

[0346] Alternatively, the production of the compounds can be achieved in a 42L fermentor system using strain NPS21184. Strain NPS21184 was grown in a 500-mL flask containing 100 mL of vegetative medium consisting of the following per liter of deionized water: glucose, 8 g; yeast extract, 6 g; Hy-Soy, 6 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28°C for 3 days on a rotary shaker operating at 250 rpm. Five mL of the first seed culture was inoculated into 500-mL flask containing of 100 mL of the vegetative medium. The second seed cultures were incubated at 28°C and 250 rpm on a rotary shaker for 2 days. Twenty mL each of the second seed culture was inoculated into 2.8 L Fernbach flask containing of 400 mL of the vegetative medium. The third seed cultures were incubated at 28°C and 250 rpm on a rotary shaker for 2 days. 1.2 L of the third seed culture was inoculated into a 42 L fermentor containing 26 L of Production Medium A. Production Medium B and Production Medium C, with the following composition, can also be used. Production Medium C consisting of the following per liter of deionized water: starch, 15 g; yeast extract 6 g; Hy-Soy, 6 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The fermentor cultures were operated at the following parameters: temperature, 28°C; agitation, 200 rpm; aeration, 13 L/min and back pressure, 4.5 psi. At 36 to 44 hours of the production cycle, approximately 600 grams of sterile Amberlite XAD-7 resin were added to the fermentor culture. The production culture was further incubated at the above operating parameters until day 4 of the production cycle. The aeration rate was lowered to 8 L/min. At day 5 of the production cycle, the fermentor culture achieved a titer of about 300 mg/L for Compound II-16. The culture broth was filtered through cheese cloth to recover the Amberlite XAD-7 resin. The resin was extracted with 2 times 4.5 liters ethyl acetate followed by 1 time 1.5 liters ethyl acetate. The combined extracts were dried in vacuo. The dried extract was then processed for the recovery of the Compounds of Formulae I-7, II-16, II-17, II-20, II-24C, II-26 and II-28.

EXAMPLE 3

PURIFICATION OF COMPOUND OF FORMULAE I-7, II-16, II-20, II-24C, II-26 AND II-

28

3A: Purification of Compound of Formulae II-16, II-20, II-24C, II-26 and II-28

[0347] The pure compounds of Formulae II-16, II-20, II-24C, II-26 and II-28 were obtained by flash chromatography followed by HPLC. Eight grams crude extract containing 3.8 grams of the compound of Formula II-16 and lesser quantities of II-20, II-24C, II-26 and II-28 was processed by flash chromatography using Biotage Flash40i system and Flash 40M cartridge (KP-Sil Silica, 32-63 μ m, 90 grams). The flash chromatography was developed by the following step gradient:

1. Hexane (1L)
2. 10% Ethyl acetate in hexane (1 L)
3. 20% Ethyl acetate in hexane, first elution (1 L)
4. 20% Ethyl acetate in hexane, second elution (1 L)
5. 20% Ethyl acetate in hexane, third elution (1 L)
6. 25% Ethyl acetate in hexane (1 L)
7. 50% Ethyl acetate in hexane (1 L)
8. Ethyl acetate (1 L)

[0348] Fractions containing the compound of Formula II-16 in greater or equal to 70% UV purity by HPLC were pooled and subject to HPLC purification, as described below, to obtain II-16, along with II-20 and II-24C, each as pure compounds

Column	Phenomenex Luna 10 μ m Silica
Dimensions	25 cm X 21.2 mm ID
Flow rate	25 mL/min
Detection	ELSD
Solvent	Gradient of 24% EtOAc/hexane for 19 min, 24% EtOAc/hexane to 100%EtOAc in 1 min, then 100% EtOAc for 4 min

[0349] The fraction enriched in compound of Formula II-16 (described above; ~ 70% pure with respect to II-16) was dissolved in acetone (60 mg/mL). Aliquots (950 μ L) of this solution were injected onto a normal-phase HPLC column using the conditions described above. Compound II-16 typically eluted after 14 minutes and compounds II-24C and II-26 co-eluted as a single peak at 11 min. When parent samples containing compounds II-17, II-20 and II-28 were processed, compound II-17 eluted at 22 minutes, while II-20 and II-28 co-eluted at 23 minutes during the 100% ethyl acetate wash. Fractions containing compound II-16 and minor analogs were pooled based on composition of compounds present, and evaporated under reduced pressure on a rotary evaporator. This process yielded pure Compound A, as well as separate fractions containing minor compounds II-20, II-24C, II-26 and II-28, which were further purified as described below.

[0350] Sample containing II-24C and II-26 generated from the process described above were further separated using reversed-phase preparative HPLC as follows. The sample containing II-24C (70 mg) was dissolved in acetonitrile at a concentration of 10 mg/mL, and 500 μ L was loaded on an HPLC column of dimensions 21 mm i.d. by 15 cm length containing Eclipse XDB-C18 support. The solvent gradient increased linearly from 15% acetonitrile /85% water to 100% acetonitrile over 23 minutes at a flow rate of 14.5 mL/min. The solvent composition was held at 100% acetonitrile for 3 minutes before returning to the starting solvent mixture. Compound II-26 eluted at 17.5 minutes while compound II-24C eluted at 19 minutes under these conditions.

[0351] Crystalline II-26 was obtained using a vapor diffusion method. Compound II-26 (15 mg) was dissolved in 100 μ L of acetone in a 1.5 mL v-bottom HPLC vial. This vial was then placed inside a larger sealed vessel containing 1 mL of pentane. Crystals suitable for X-ray crystallography experiments were observed along the sides and bottom of the inner vial after 48 hours of incubation at 4°C. Crystallography data was collected on a Bruker SMART APEX CCD X-ray diffractometer ($F(000)=2656$, MoK_{α} radiation, $\lambda=0.71073$ Å, $\mu=0.264$ mm⁻¹, $T=100$ K) at the UCSD Crystallography Lab and the refinement method used was full-matrix least-squares on F^2 . Crystal data NPI-2065: $C_{15}H_{20}ClNO_4$, $MW=313.77$, tetragonal, space group $P4(1)2(1)2$, $a= b=11.4901(3)$ Å, $c= 46.444(2)$ Å, $\alpha=\beta=\gamma=90^\circ$,

$\text{vol}=6131.6(3) \text{ \AA}^3$, $Z=16$, $\rho_{\text{calc}}=1.360 \text{ g cm}^{-3}$, crystal size, $0.30 \times 0.15 \times 0.07 \text{ mm}^3$, θ range, $1.75\text{--}26.00^\circ$, 35367 reflections collected, 6025 independent reflections ($R_{\text{int}}=0.0480$), final R indices ($I > 2\sigma(I)$): $R_1=0.0369$, $wR_2=0.0794$, $\text{GOF}=1.060$.

[0352] In order to separate II-28 from II-20, a reverse-phase isocratic method was employed. Sample (69.2 mg) containing both compounds was dissolved in acetonitrile to a concentration of 10 mg/mL, and 500 μL was loaded on a reverse-phase HPLC column (ACE 5 C18-HL, 15 cm X 21 mm ID) per injection. An isocratic solvent system of 27% acetonitrile/ 63% water at flow rate of 14.5 mL/min was used to separate compounds II-28 and II-20, which eluted after 14 and 16 minutes, respectively. Fractions containing compounds of interest were immediately evaporated under reduced pressure at room temperature on a rotary evaporator. Samples were then loaded onto a small column of silica and eluted with 10 mL of 70% hexane/30% acetone to remove additional impurities.

[0353] Samples generated from the preparative normal-phase HPLC method described above that contained II-20, but which were free of II-28 could also be triturated with 100% EtOAc to remove minor lipophilic impurities.

[0354] Compound of Formula II-16: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 314 (M+H), 336 (M+Na).

[0355] Compound of Formula II-20: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 266 (M+H); HRMS (ESI), m/z 266.1396 (M+H), $\Delta_{\text{calc}} = 1.2 \text{ ppm}$.

[0356] Compound of Formula II-24C: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 328 (M+H), 350 (M+Na); HRMS (ESI), m/z 328.1309 (M+H), $\Delta_{\text{calc}} = -2.0 \text{ ppm}$, $\text{C}_{16}\text{H}_{23}\text{NO}_4\text{Cl}$.

[0357] Compound of Formula II-26: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm; HRMS (ESI), m/z 314.1158 (M+H), $\Delta_{\text{calc}} = -0.4 \text{ ppm}$, $\text{C}_{15}\text{H}_{21}\text{NO}_4\text{Cl}$.

[0358] Compound of Formula II-28: UV (Acetonitrile/ H_2O) λ_{max} 225(sh) nm; HRMS (ESI), m/z 266.1388 (M+H), $\Delta_{\text{calc}} = -1.8 \text{ ppm}$, $\text{C}_{14}\text{H}_{20}\text{NO}_4$.

3B: Purification of Compound of Formula I-7

[0359] A Biotage Flash 75Li system with a Flash 75L KP-Sil cartridge was used to process the filtered crude extract (10.0 g), enriched in Compound II-16 and containing

Compound of Formula I-7. The crude extract was dissolved to a concentration of 107 mg/mL in acetone and loaded directly onto the cartridge. The following solvent step gradient was then run through the cartridge at a flow rate between 235 mL/min and 250 mL/min

1. 10% EtOAc in n-Heptane (3.2 L)
2. 25% EtOAc in n-Heptane (16 L)
3. 30% EtOAc in n-Heptane (5.4 L)

[0360] Fractions enriched in Compound II-16 were pooled and concentrated by rotavapor until ~ 5% of the total pooled volume of solvent remained. The solvent was removed, leaving behind the white solid.

[0361] A crystallization was then performed on the solid by dissolving the sample (4.56 g) in 1:1 acetone:n-heptane (910 mL). The solvent was slowly evaporated using a rotary evaporator until the solvent was reduced to about 43% of its original volume. The solution (supernatant) was removed and concentrated (598 mg).

[0362] The supernatant was dissolved in acetone (80 mg/mL). Aliquots (500 μ L) of this solution were injected onto a normal-phase HPLC column using the conditions described above for normal phase purification of Compounds II-16, II-24C, II-26 and II-28. Compound of Formula I-7 eluted at 7.5 minutes as a pure compound.

[0363] Compound of Formula I-7 (FIG. 58): UV (Acetonitrile/H₂O) λ_{max} 225(sh) nm. Low Res. Mass: m/z 298 (M+H), 320 (M+Na).

EXAMPLE 4

FERMENTATION OF COMPOUNDS OF FORMULAE II-17, II-18, AND II-27

[0364] Strain CNB476 was grown in a 500-mL flask containing 100 mL of the first vegetative medium consisting of the following per liter of deionized water: glucose, 4 g; Bacto tryptone, 3 g; Bacto casitone, 5 g; and synthetic sea salt (Instant Ocean, Aquarium Systems), 30 g. The first seed culture was incubated at 28 C for 3 days on a rotary shaker operating at 250 rpm. Five mL of the first seed culture was inoculated into a 500-mL flask containing 100 mL of the second vegetative medium consisting of the following per liter of deionized water: starch, 10 g; yeast extract, 4 g; peptone, 2 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and sodium bromide, 30 g. The second seed

cultures were incubated at 28°C for 7 days on a rotary shaker operating at 250 rpm. Approximately 2 to 3 gram of sterile Amberlite XAD-7 resin were added to the second seed culture. The second seed culture was further incubated at 28°C for 2 days on a rotary shaker operating at 250 rpm. Five ml of the second seed culture was inoculated into a 500-ml flask containing 100 mL of the second vegetative medium. The third seed culture was incubated at 28°C for 1 day on a rotary shaker operating at 250 rpm. Approximately 2 to 3 gram of sterile Amberlite XAD-7 resin were added to the third seed culture. The third seed culture was further incubated at 28°C for 2 days on a rotary shaker operating at 250 rpm. Five ml of the third culture was inoculated into a 500-ml flask containing 100 mL of the second vegetative medium. The fourth seed culture was incubated at 28°C for 1 day on a rotary shaker operating at 250 rpm. Approximately 2 to 3 gram of sterile Amberlite XAD-7 resin were added to the fourth seed culture. The fourth seed culture was further incubated at 28°C for 1 day on a rotary shaker operating at 250 rpm. Five mL each of the fourth seed culture was inoculated into ten 500-mL flasks containing 100 mL of the second vegetative medium. The fifth seed cultures were incubated at 28°C for 1 day on a rotary shaker operating at 250 rpm. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were added to the fifth seed cultures. The fifth seed cultures were further incubated at 28°C for 3 days on a rotary shaker operating at 250 rpm. Four mL each of the fifth seed culture was inoculated into one hundred and fifty 500-mL flasks containing 100 mL of the production medium having the same composition as the second vegetative medium. Approximately 2 to 3 grams of sterile Amberlite XAD-7 resin were also added to the production culture. The production cultures were incubated at 28°C for 6 day on a rotary shaker operating at 250 rpm. The culture broth was filtered through cheese cloth to recover the Amberlite XAD-7 resin. The resin was extracted with 2 times 3 liters ethyl acetate followed by 1 time 1 liter ethyl acetate. The combined extracts were dried *in vacuo*. The dried extract, containing 0.42 g of the compound Formula II-17 and 0.16 gram the compound of Formula II-18, was then processed for the recovery of the compounds.

EXAMPLE 5

PURIFICATION OF COMPOUNDS OF FORMULA II-17, II-18 AND II-27

[0365] The pure compounds of Formula II-17 and II-18 were obtained by reversed-phase HPLC as described below:

Column	ACE 5 C18-HL
Dimensions	15 cm X 21 mm ID
Flow rate	14.5 mL/min
Detection	214 nm
Solvent	Gradient of 35% acetonitrile/65% H ₂ O to 90% acetonitrile/10% H ₂ O over 15 min

[0366] Crude extract (100 mg) was dissolved in 15 mL of acetonitrile. Aliquots (900 µL) of this solution were injected onto a reversed-phase HPLC column using the conditions described above. Compounds of Formulae II-17 and II-18 eluted at 7.5 and 9 minutes, respectively. Fractions containing the pure compounds were first concentrated using nitrogen to remove organic solvent. The remaining solution was then frozen and lyophilized to dryness.

[0367] An alternative purification method for Compound II-17 and II-18 was developed for larger scale purification and involved fractionation of the crude extract on a normal phase VLC column. Under these conditions, sufficient amounts of several minor metabolites were identified, including compound II-27. The crude extract (2.4 g) was dissolved in acetone (10 mL) and this solution adsorbed onto silica gel (10 cc) by drying *in vacuo*. The adsorbed crude extract was loaded on a normal phase silica VLC column (250 cc silica gel, column dimensions 2.5 cm diameter by 15 cm length) and washed with a step gradient of hexane / EtOAc, increasing in the percentage of hexane in steps of 5% (100 mL solvent per step). The majority of compound II-16 eluted in the 60% hexane / 40% EtOAc wash while the majority of compound II-17 eluted in the 50% hexane / 50% ethyl acetate wash. Final separation of the compounds was achieved using C18 HPLC chromatography (ACE 5 µm C18-HL, 150 mm X 21 mm ID) using an isocratic solvent system consisting of

35% acetonitrile/65% H₂O. Under these conditions, compound II-27 eluted at 11 minutes, compound II-17 eluted at 12.0 minutes, traces of compound A eluted at 23.5 minutes, and compound II-18 eluted at 25.5 minutes. The resulting samples were dried *in vacuo* using no heat to remove the aqueous solvent mixture. The spectroscopic data for these samples of compound II-16 and compound II-18 were found to be identical with those of samples prepared from earlier purification methods. The sample of compound II-18 was found to contain 8% of the lactone hydrolysis product and was further purified by washing through a normal phase silica plug (1 cm diameter by 2 cm height) and eluting using a solvent mixture of 20% EtOAc / 80% Hexanes (25 mL). The resulting sample was found to contain pure compound II-18.

[0368] The fractions containing compound II-27 described above were further purified using normal phase semipreparative HPLC (Phenomenex Luna Si 10 μ m, 100 Å, 250 x 10 mm id) using a solvent gradient increasing from 100% hexane to 100% EtOAc over 20 minutes with a flowrate of 4 mL/min. Compound II-27 eluted as a pure compound after 11.5 minutes (0.8 mg, 0.03% isolated yield from dried extract weight).

[0369] Compound of Formula II-17: UV (Acetonitrile/H₂O) λ_{\max} 225(sh) nm. High Res. Mass (APCI): m/z 280.156 (M+H), Δ_{calc} =2.2 ppm, C₁₅H₂₂NO₄.

[0370] Compound of Formula II-18: UV (Acetonitrile/H₂O) λ_{\max} 225(sh) nm. High Res. Mass (APCI): m/z 358.065 (M+H), Δ_{calc} = -1.9 ppm, C₁₅H₂₁NO₄Br.

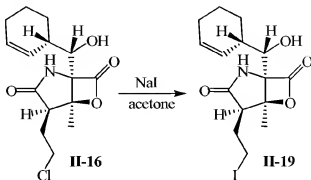
[0371] Compound II-27: UV (Acetonitrile/H₂O) λ_{\max} 225(sh) nm; MS (HR-ESI), m/z 280.1556 (M+H) Δ_{calc} = 2.7 ppm (C₁₅H₂₂NO₄).

EXAMPLE 6

PREPARATION OF COMPOUND OF FORMULA II-19 FROM II-16

[0372] A sample of compound of Formula II-16 (250 mg) was added to an acetone solution of sodium iodide (1.5 g in 10 mL) and the resulting mixture stirred for 6 days. The solution was then filtered through a 0.45 micron syringe filter and injected directly on a normal phase silica HPLC column (Phenomenex Luna 10 μ m Silica, 25 cm x 21.2 mm) in 0.95 mL aliquots. The HPLC conditions for the separation of compound formula II-19 from unreacted II-16 employed an isocratic HPLC method consisting of 24% ethyl acetate

and 76% hexane, in which the majority of compound II-19 eluted 2.5 minutes before compound II-16. Equivalent fractions from each of 10 injections were pooled to yield 35 mg compound II-19. Compound II-19: UV (Acetonitrile/H₂O) 225 (sh), 255 (sh) nm; ESMS, m/z 406.0 (M+H); HRMS (ESI), m/z 406.0513 [M+H]⁺, $\Delta_{\text{calc}} = -0.5$ ppm, C₁₅H₂₁NO₄I.

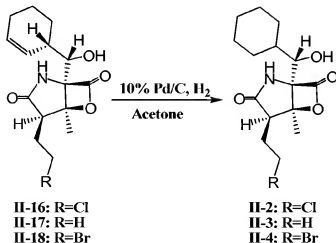


EXAMPLE 7

SYNTHESIS OF THE COMPOUNDS OF FORMULAE II-2, II-3, AND II-4

[0373] Compounds of Formulae II-2, II-3 and II-4 can be synthesized from compounds of Formulae II-16, II-17 and II-18, respectively, by catalytic hydrogenation.

Exemplary Depiction of Synthesis



Example 7A: Catalytic Hydrogenation of Compound of Formula II-16

[0374] Compound of Formula II-16 (10 mg) was dissolved in acetone (5 mL) in a scintillation vial (20 mL) to which was added the 10% (w/w) Pd/C (1-2 mg) and a magnetic

stirrer bar. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 3 cc silica column and washed with acetone. The filtrate was filtered again through 0.2 μ m Gelman Acrodisc to remove any traces of catalyst. The solvent was evaporated off from filtrate under reduced pressure to yield the compound of Formula II-2 as a pure white powder: UV (acetonitrile/H₂O): λ_{max} 225 (sh) nm; m/z 316 (M+H), 338 (M+Na).

Example 7B: Catalytic Hydrogenation of Compound of Formula II-17

[0375] Compound of Formula II-17 (5 mg) was dissolved in acetone (3 mL) in a scintillation vial (20 mL) to which was added the 10% (w/w) Pd/C (about 1 mg) and a magnetic stirrer bar. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodisc to remove the catalyst. The solvent was evaporated off from filtrate to yield the compound of Formula II-3 as a white powder which was purified by normal phase HPLC using the following conditions:

Column:	Phenomenex Luna 10 μ m Silica
Dimensions:	25 cm x 21.2 mm ID
Flow rate:	14.5 mL/min
Detection:	ELSD
Solvent:	5% to 60% EtOAc/Hex for 19 min, 60 to 100% EtOAc in 1 min, then 4 min at 100% EtOAc

[0376] Compound of Formula II-3 eluted at 22.5 min as a pure compound: UV (acetonitrile/H₂O): λ_{max} 225 (sh) nm; m/z 282 (M+H), 304 (M+Na).

Example 7C: Catalytic Hydrogenation of Compound of Formula II-18

[0377] 3.2 mg of compound of Formula II-18 was dissolved in acetone (3 mL) in a scintillation vial (20 mL) to which was added the 10% (w/w) Pd/C (about 1 mg) and a magnetic stirrer bar. The reaction mixture was stirred in hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman

Acrodisc to remove the catalyst. The solvent was evaporated off from filtrate to yield the compound of Formula II-4 as a white powder which was further purified by normal phase HPLC using the following conditions:

Column:	Phenomenex Luna 10 μ m Silica
Dimensions:	25 cm x 21.2 mm ID
Flow rate:	14.5 mL/min
Detection:	ELSD
Solvent:	5% to 80% EtOAc/Hex for 19 min, 80 to 100% EtOAc in 1 min, then 4 min at 100% EtOAc

[0378] Compound of Formula II-4 eluted at 16.5 min as a pure compound: UV (acetonitrile/H₂O): λ_{max} 225 (sh) nm; m/z 360 (M+H), 382 (M+Na).

[0379] In addition, high resolution mass spectrometry data were obtained for compounds II-2, II-3, and II-4. Compound II-2: HRMS (ESI), m/z 316.1305 [M+H]⁺, Δ_{calc} = -3.5 ppm, C₁₅H₂₃NO₄Cl. Compound II-3: HRMS (ESI), m/z 282.1706 [M+H]⁺, Δ_{calc} = 0.3 ppm, C₁₅H₂₄NO₄. Compound II-4: HRMS (ESI), m/z 360.0798 [M+H]⁺, Δ_{calc} = -3.4 ppm, C₁₅H₂₃NO₄Br.

EXAMPLE 8

SYNTHESIS OF THE COMPOUNDS OF FORMULAE II-5A AND II-5B

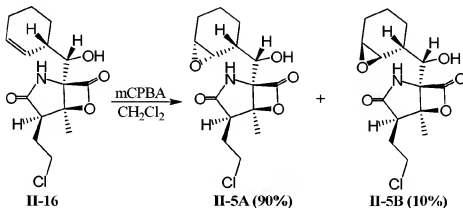
[0380] Compounds of Formula II-5A and Formula II-5B can be synthesized from compound of Formula II-16 by epoxidation with mCPBA.

[0381] Compound of Formula II-16 (101 mg, 0.32 mmole) was dissolved in methylenechloride (30 mL) in a 100 mL of round bottom flask to which was added 79 mg (0.46 mmole) of meta-chloroperbenzoic acid (mCPBA) and a magnetic stir bar. The reaction mixture was stirred at room temperature for about 18 hours. The reaction mixture was poured onto a 20 cc silica flash column and eluted with 120 mL of CH₂Cl₂, 75 mL of 1:1 ethyl acetate/hexane and finally with 40 mL of 100% ethyl acetate. The 1:1 ethyl acetate/hexane fractions yield a mixture of diastereomers of epoxyderivatives, Formula II-5A and II-5B, which were separated by normal phase HPLC using the following conditions:

Column	Phenomenex Luna 10 μm Silica
Dimensions	25 cm x 21.2 mm ID
Flow rate	14.5 mL/min
Detection	ELSD
Solvent	25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc in 1 min, then 5 min at 100% EtOAc

[0382] Compound Formula II-5A (major product) and II-5B (minor product) eluted at 21.5 and 19 min, respectively, as pure compounds. Compound II-5B was further chromatographed on a 3 cc silica flash column to remove traces of chlorobenzoic acid reagent.

Chemical Structures:



Structural Characterization

[0383] Formula II-5A: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm. Low Res. Mass: m/z 330 (M+H), 352 (M+Na); HRMS (ESI), m/z 330.1099 [M+H]⁺, Δ_{calc} = -2.9 ppm, C₁₅H₂₁NO₅Cl.

[0384] Formula II-5B: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm. Low Res. Mass: m/z 330 (M+H), 352 (M+Na); HRMS (ESI), m/z 330.1105 [M+H]⁺, Δ_{calc} = -0.9 ppm, C₁₅H₂₁NO₅Cl.

EXAMPLE 9

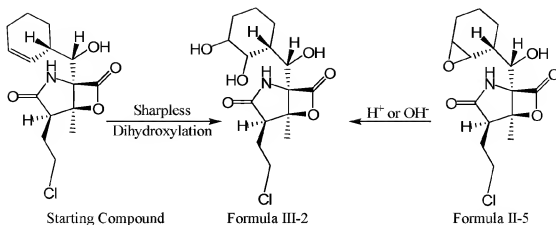
SYNTHESIS OF THE COMPOUNDS OF FORMULAE III-1, III-2, III-3 AND III-4

Synthesis of diol derivatives (Formula III-2)

[0385] Diols can be synthesized by Sharpless dihydroxylation using AD mix- α and β : AD mix- α is a premix of four reagents, K₂OsO₂(OH)₄; K₂CO₃; K₃Fe(CN)₆; (DHQ)-PHAL [1,4-bis(9-O-dihydroquinine)phthalazine] and AD mix- β is a premix of K₂OsO₂(OH)₄; K₂CO₃; K₃Fe(CN)₆; (DHQD)₂-PHAL [1,4-bis(9-O-dihydroquinidine)phthalazine] which are commercially available from Aldrich. The diol can also be synthesized by acid or base hydrolysis of epoxy compounds (Formula II-5A and II-5B) which may be different to that of products obtained in Sharpless dihydroxylation in their stereochemistry at carbons bearing hydroxyl groups

Sharpless Dihydroxylation of Compounds II-16, II-17 and II-18

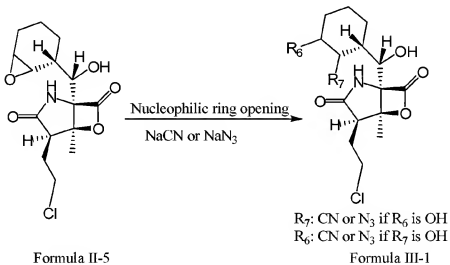
[0386] Any of the compounds of Formulae II-16, II-17 and II-18 can be used as the starting compound. In the example below, compound of Formula II-16 is used. The starting compound is dissolved in t-butanol/water in a round bottom flask to which is added AD mix- α or β and a magnetic stir bar. The reaction is monitored by silica TLC as well as mass spectrometer. The pure diols are obtained by usual workup and purification by flash chromatography or HPLC. The structures are confirmed by NMR spectroscopy and mass spectrometry. In this method both hydroxyl groups are on same side.



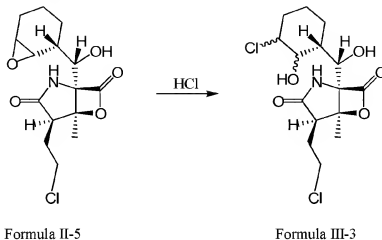
Nucleophilic ring opening of epoxy compounds (II-5):

[0387] The epoxy ring is opened with various nucleophiles like NaCN, NaN₃, NaOAc, HBr, HCl, etc to creat various substituents on the cyclohexane ring, including a hydroxyl substituent.

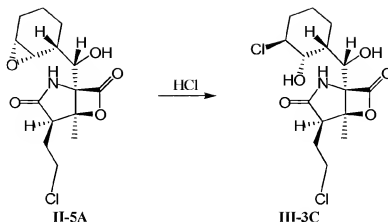
Examples:



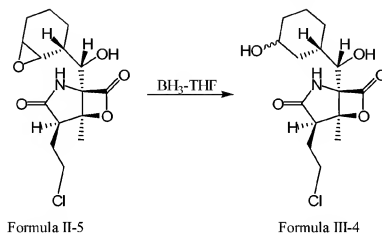
[0388] The epoxy is opened with HCl to make Formula III-3:



[0389] Compound of Formula II-5A (3.3 mg) was dissolved in acetonitrile (0.5 mL) in a 1 dram vial to which was added 5% HCl (500 μ L) and a magnetic stir bar. The reaction mixture was stirred at room temperature for about an hour. The reaction was monitored by mass spectrometry. The reaction mixture was directly injected on normal phase HPLC to obtain compound of Formula III-3C as a pure compound without any work up. The HPLC conditions used for the purification were as follows: Phenomenex Luna 10 μ m Silica column (25 cm x 21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc in 1 min, then 5 min at 100% EtOAc at a flow rate of 14.5 mL/min. An ELSD was used to monitor the purification process. Compound of Formula III-3C eluted at about 18 min (2.2 mg). Compound of Formula III-3C: UV (Acetonitrile/ H_2O) λ_{max} 225 (sh) nm; ESMS, m/z 366 (M+H), 388 (M+Na); HRMS (ESI), m/z 366.0875 [M+H]⁺, Δ_{calc} = 0.0 ppm, $C_{15}H_{22}NO_5Cl_2$. The stereochemistry of the compound of Formula III-3C was determined based on coupling constants observed in the cyclohexane ring in 1:1 C_6D_6 /DMSO- d_6 .



[0390] Reductive ring opening of epoxides (II-5): The compound of Formula is treated with metalhydrides like $\text{BH}_3\text{-THF}$ complex to make compound of Formula III-4.



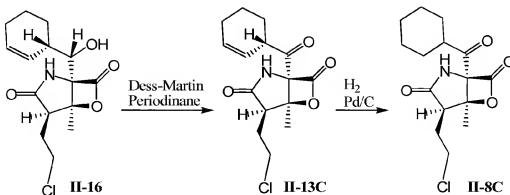
EXAMPLE 10

SYNTHESIS OF THE COMPOUNDS OF FORMULAE II-13C AND II-8C

[0391] Compound of Formula II-16 (30 mg) was dissolved in CH_2Cl_2 (6 mL) in a scintillation vial (20 mL) to which Dess-Martin Periodinane (122 mg) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 2 hours. The progress of the reaction was monitored by TLC (Hex:EtOAc, 6:4) and analytical HPLC. From the reaction mixture, the solvent volume was reduced to one third, absorbed on silica gel, poured on top of a 20 cc silica flash column and eluted in 20 mL fractions using a gradient of Hexane/EtOAc from 10 to 100%. The fraction eluted with 30% EtOAc in Hexane

contained a mixture of rotamers of Formula II-13C in a ratio of 1.5:8.5. The mixture was further purified by normal phase HPLC using the Phenomenex Luna 10 μ m Silica column (25 cm x 21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc over 1 min, holding at 100% EtOAc for 5 min, at a flow rate of 14.5 mL/min. An ELSD was used to monitor the purification process. Compound of Formula II-13C eluted at 13.0 and 13.2 mins as a mixture of rotamers with in a ratio of 1.5:8.5 (7 mg). Formula II-13C: UV (Acetonitrile/H₂O) λ_{max} 226 (sh) & 300 (sh) nm; ESMS, m/z 312 (M+H)⁺, 334 (M+Na)⁺; HRMS (ESI), m/z 312.1017 [M+H]⁺, Δ_{calc} = 4.5 ppm, C₁₅H₁₉NO₄Cl.

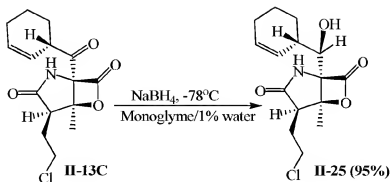
[0392] The rotamer mixture of Formula II-13C (4 mg) was dissolved in acetone (1 mL) in a scintillation vial (20 mL) to which a catalytic amount (0.5 mg) of 10% (w/w) Pd/C and a magnetic stir bar were added. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodisc to remove the catalyst. The solvent was evaporated from the filtrate to yield compound of Formula II-8C as a colorless gum which was further purified by normal phase HPLC using a Phenomenex Luna 10 μ m Silica column (25 cm x 21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc over 1 min, holding at 100% EtOAc for 5 min, at a flow rate of 14.5 mL/min. An ELSD was used to monitor the purification process. Compound of Formula II-8C (1 mg) eluted at 13.5 min as a pure compound. Formula II-8C: UV (Acetonitrile/H₂O) λ_{max} 225 (sh) nm; ESMS, m/z 314 (M+H)⁺, 336 (M+Na)⁺; HRMS (ESI), m/z 314.1149 [M+H]⁺, Δ_{calc} = 3.3 ppm, C₁₅H₂₁NO₄Cl.



EXAMPLE 11

SYNTHESIS OF THE COMPOUND OF FORMULA II-25 FROM II-13C

[0393] The rotamer mixture of Formula II-13C (5 mg) was dissolved in dimethoxy ethane (monoglyme; 1.5 mL) in a scintillation vial (20 mL) to which water (15 μ L (1% of the final solution concentration)) and a magnetic stir bar were added. The above solution was cooled to -78°C on a dry ice-acetone bath, and a sodium borohydride solution (3.7 mg of NaBH_4 in 0.5 mL of monoglyme (created to allow for slow addition)) was added drop-wise. The reaction mixture was stirred at -78°C for about 14 minutes. The reaction mixture was acidified using 2 mL of 4% HCl solution in water and extracted with CH_2Cl_2 . The organic layer was evaporated to yield mixture of compound of formulae II-25 and II-16 in a 9.5:0.5 ratio as a white solid, which was further purified by normal phase HPLC using a Phenomenex Luna 10 μ m Silica column (25 cm x 21.2 mm ID). The mobile phase was 24% EtOAc/76% Hexane, which was held isocratic for 19 min, followed by a linear gradient of 24% to 100% EtOAc over 1 min, and held at 100% EtOAc for 3 min; the flow rate was 25 mL/min. An ELSD was used to monitor the purification process. Compound of formula II-25 (1.5 mg) eluted at 11.64 min as a pure compound. Compound of Formula II-25: UV (Acetonitrile/ H_2O) λ_{max} 225 (sh) nm; ESMS, m/z 314 ($\text{M}+\text{H}$) $^+$, 336 ($\text{M}+\text{Na}$) $^+$; HRMS (ESI), m/z 314.1154 [$\text{M}+\text{H}$] $^+$, Δ_{calc} = -0.6 ppm, $\text{C}_{15}\text{H}_{21}\text{NO}_4\text{Cl}$.



EXAMPLE 12

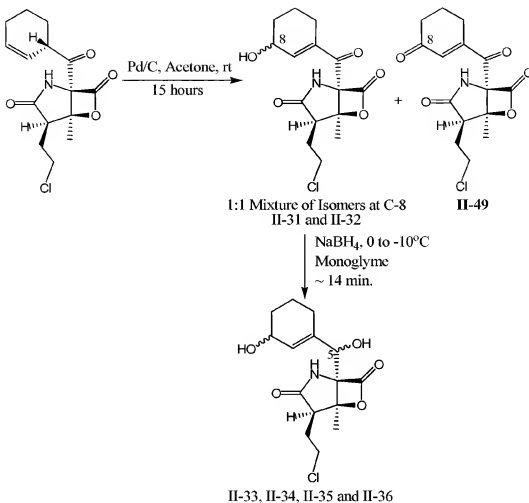
SYNTHESIS OF THE COMPOUNDS OF FORMULAE II-31, II-32 AND II-49 FROM II-13C; AND COMPOUNDS OF FORMULAE II-33, II-34, II-35 AND II-36 FROM II-31

AND II-32

[0394] A rotamer mixture of the Compound of Formula II-13C (20 mg) was dissolved in acetone (4 mL) in a scintillation vial (20 mL) to which a catalytic amount (3 mg) of 10% (w/w) Pd/C and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 15 hours. The reaction mixture was filtered through a 0.2 μ m Gelman Acrodisc to remove the catalyst. The solvent was evaporated from the filtrate to yield a mixture of diastereomers of hydroxy derivatives of Formulae II-31 and II-32 (1:1) and a minor compound II-49, which were separated by reversed phase HPLC using Ace 5 μ m C18 column (150 mm x 22 mm ID) with a solvent gradient of 90% to 30% H₂O/acetonitrile over 15 min, 70 to 100% acetonitrile over 5 min, holding at 100% acetonitrile for 4 min, at a flow rate of 14.5 mL/min. A diode array detector was used to monitor the purification process. Compound II-31 (2 mg), II-32 (2 mg) and II-49 (0.2 mg) eluted at 10.6, 10.8 and 11.54 min, respectively, as pure compounds. II-31: UV (Acetonitrile/H₂O) λ_{max} 250 (sh) nm; ESMS m/z 328.1 (M+H)⁺ & 350.0 (M+Na)⁺. II-32: UV (Acetonitrile/H₂O) λ_{max} 250 (sh) nm; ESMS, m/z 328.1 (M+H)⁺ & 350.0 (M+Na)⁺. II-49: UV (Acetonitrile/H₂O) λ_{max} 250 (sh) and 320 nm; ESMS, m/z 326.0 (M+H)⁺, 343.1 (M+H₂O)⁺ & 348.0 (M+Na)⁺.

[0395] In an alternate method, compounds II-31, II-32 and II-49 were separated by normal phase HPLC using Phenomenex Luna 10 μ m Silica column (25 cm x 21.2 mm ID) with a solvent gradient of 10% to 100% Hexane/EtOAc over 24 min, holding at 100% EtOAc for 3 min, at a flow rate of 14.5 mL/min. ELSD was used to monitor the purification process.

[0396] The ketone of the compounds of formula II-31 and II-32 can be reduced by using sodium borohydride at 0 to -10°C in monoglyme solvent for about 14 minutes. The reaction mixture can be acidified using 4% HCl solution in water and extracted with CH₂Cl₂. The organic layer can be evaporated to yield the mixtures of compounds of formulae II-33, II-34, II-35 and II-36 which can be separated by chromatographic methods.

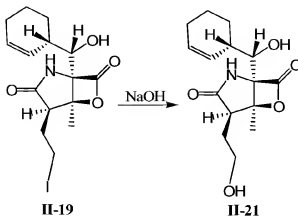


EXAMPLE 13

SYNTHESIS OF THE COMPOUND OF FORMULAE II-21 FROM II-19

[0397] Acetone (7.5 mL) was vigorously mixed with 5 N NaOH (3 mL) and the resulting mixture evaporated to a minimum volume *in vacuo*. A sample of 100 μL of this solution was mixed with compound of Formula II-19 (6.2 mg) in acetone (1 mL) and the resulting biphasic mixture vortexed for 2 minutes. The reaction solution was immediately subjected to preparative C18 HPLC. Conditions for the purification involved a linear gradient if 10% acetonitrile/90% water to 90% acetonitrile/ 10% water over 17 minutes using an Ace 5 μm C18 HPLC column of dimensions 22 mm id by 150 mm length. Compound of

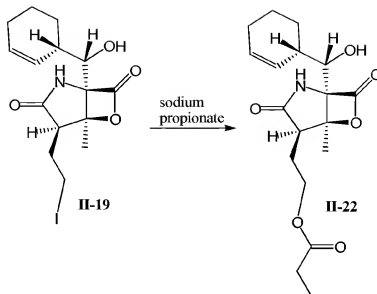
Formula II-21 eluted at 9.1 minutes under these conditions to yield 0.55 mg compound.
Compound of Formula II-21: UV (Acetonitrile/H₂O) 225 (sh), ESMS, m/z 296.1 (M+H).



EXAMPLE 14

SYNTHESIS OF THE COMPOUND OF FORMULAE II-22 FROM II-19

[0398] A sample of 60 mg sodium propionate was added to a solution of compound of Formula II-19 (5.3 mg) in DMSO (1 mL) and the mixture sonicated for 5 minutes, though the sodium propionate did not completely dissolve. After 45 minutes, the solution was filtered through a 0.45 μm syringe filter and purified directly using HPLC. Conditions for the purification involved a linear gradient if 10% acetonitrile/90% water to 90% acetonitrile/10% water over 17 minutes using an Ace 5 μm C18 HPLC column of dimensions 22 mm id by 150 mm length. Under these conditions, compound of Formula II-22 eluted at 12.3 minutes to yield 0.7 mg compound (15% isolated yield). UV (Acetonitrile/H₂O) 225 (sh), ESMS, m/z 352.2 (M+H); HRMS (ESI), m/z 352.1762 [M+H]⁺, Δ_{calc} = 0.6 ppm, C₁₈H₂₆NO₆.

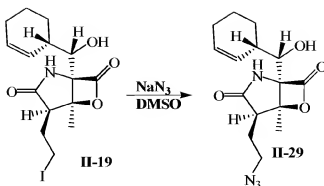


EXAMPLE 15

SYNTHESIS OF THE COMPOUND OF FORMULA II-29 FROM II-19

[0399] A sample of NaN_3 (80 mg) was dissolved in DMSO (1 mL) and transferred to a vial containing Compound II-19 (6.2 mg) which was contaminated with approximately 10% Compound II-16. The solution was incubated at room temperature for 1 hr prior to purification on C18 HPLC (ACE 5 μm C18-HL, 150 mm X 21 mm ID) using a solvent gradient of 10% acetonitrile/90% H_2O to 90% acetonitrile/10% H_2O over 17 minutes. Using this method, the desired azido derivative II-29 co-eluted with Compound II-16 contaminant at 12.5 minutes (4.2 mg, 85% yield). A 2.4 mg portion of compound II-29 was further purified using additional C18 HPLC chromatography (ACE 5 μm C18-HL, 150 mm X 21 mm ID) using an isocratic solvent gradient consisting of 35% acetonitrile/65% H_2O . Under these conditions compound II-29 eluted after 20 minutes, while Compound II-16 eluted after 21.5 minutes. The resulting sample consisted of 1.1 mg Compound II-29 was used for characterization in biological assays.

[0400] Compound II-29: UV (Acetonitrile/ H_2O) 225 (sh), ESMS, m/z 321.1 (M+H).



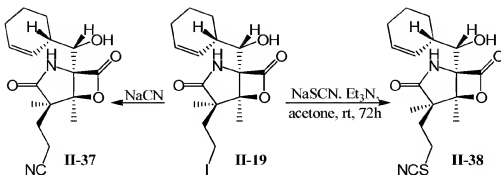
EXAMPLE 16

SYNTHESIS OF THE COMPOUNDS OF FORMULAE II-37 AND II-38 FROM II-19

[0401] The compounds of Formulae II-37 and II-38 can be prepared from the compound of Formula II-19 by cyano-de-halogenation or thiocyanato-de-halogenation, respectively. Compound II-19 can be treated with NaCN or KCN to obtain compound II-37. Alternatively, Compound II-19 can be treated with NaSCN or KSCN to obtain compound II-38.

Synthesis of the compound of Formula II-38 from II-19:

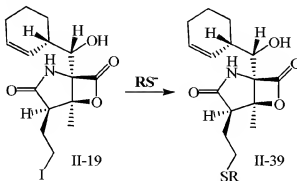
[0402] The compound of formula II-19 (10.6 mg, 0.0262 mmol) was dissolved in 1.5 mL of acetone in a scintillation vial (20 mL) to which sodium thiocyanate (10.0 mg, 0.123 mmol), triethylamine (5 μ L, 0.036 mmol) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for 72 hours. The reaction mixture was concentrated *in vacuo* to yield the compound II-38. Compound II-38 was purified by normal phase HPLC using a Phenomenex Luna 10 μ m Silica column (25cm x 21.2 mm ID) with a solvent gradient of 0 to 95% H₂O/acetonitrile over 21 min, at a flow rate of 14.5 mL/min. Diode array detector was used to monitor the purification process. Compound II-38 (3.0 mg, 34% yield) eluted at 18.0 min as a pure compound. II-38: UV Acetonitrile/H₂O λ_{max} 203 (sh) nm; ESMS m/z 337.1 (M+H)⁺ & 359.1 (M+Na)⁺.



EXAMPLE 17

SYNTHESIS OF THE COMPOUND OF FORMULA II-39 FROM II-19

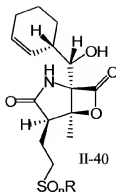
[0403] Thiols and thioethers of the Formula II-39 can be formed by dehalogenation of the compound of Formula II-19. Thiols (R=H) can be formed by treatment of Compound II-19 with NaSH, for example, while thioethers (R=alkyl) can be formed by treatment of Compound II-19 with salts of thiols, or alternatively, by treatment with thiols themselves by running the reaction in benzene in the presence of DBU.



EXAMPLE 18

SYNTHESIS OF THE COMPOUND OF FORMULA II-40 FROM II-39

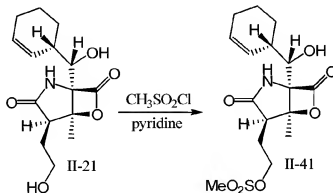
[0404] Sulfoxides (n=1) and sulfones (n=2) of the Formula II-40 can be formed by oxidation of thioethers of the Formula II-39, for example, with hydrogen peroxide or other oxidizing agents.



EXAMPLE 19

SYNTHESIS OF THE COMPOUND OF FORMULA II-41 FROM II-21

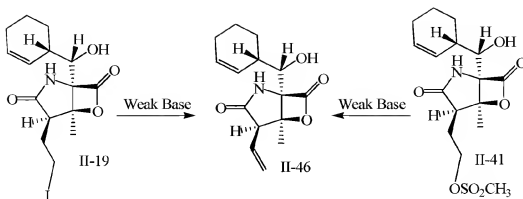
[0405] The compound of the Formula II-41 can be prepared by treatment of the compound of Formula II-21 (or a protected derivative of II-21, where the C-5 alcohol or lactam NH are protected, for example) with methyl sulfonyl chloride (mesyl chloride) in pyridine, for example, or by treatment with mesyl chloride in the presence of triethylamine. Other sulfonate esters can be similarly prepared.



EXAMPLE 20

SYNTHESIS OF THE COMPOUND OF FORMULA II-46 FROM II-19 OR II-41

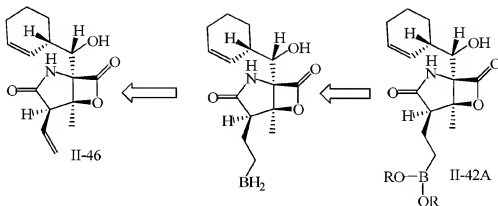
[0406] The alkene of the Formula II-46 can be prepared by dehydroiodination of the compound of Formula II-19, or by hydro-mesyloxy elimination of the compound of Formula II-41, for example, by treatment with base.



EXAMPLE 21

SYNTHESIS OF THE COMPOUND OF FORMULA II-42A

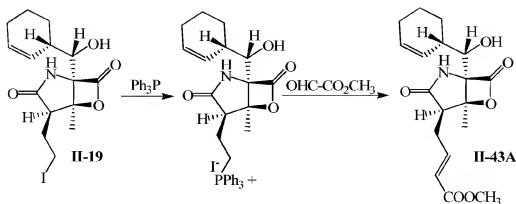
[0407] Synthesis of boronic acids or esters, for example, the compound of the Formula II-42A, can be achieved as outlined in the retrosynthetic scheme below. Hydroboration of the alkene of Formula II-46 gives the corresponding alkyl borane, which can be converted to the corresponding boronic acid or ester, for example, the compound of the Formula II-42A.



EXAMPLE 22

SYNTHESIS OF THE COMPOUND OF FORMULA II-43A

[0408] The compound of the Formula II-43A can be prepared by treatment of the compound of Formula II-19 with triphenyl phosphine to make a phosphorus ylide, which can be treated with various aldehydes, for example, glyoxylic acid methyl ester, to make Formula II-43A.

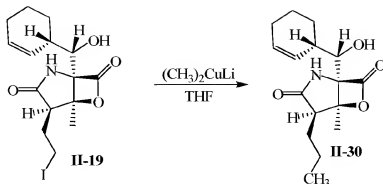


EXAMPLE 23

SYNTHESIS OF THE COMPOUND OF FORMULA II-30 FROM II-19

[0409] A portion of CuI (100 mg) was placed in a 25 mL pear bottom flask and flushed with argon gas for 30 minutes. Argon gas flow was maintained through the flask throughout the course of the reaction. The vessel was cooled to -78°C prior to addition of dry THF (5 mL) followed by the immediate dropwise addition of a solution of methyllithium in dry THF (5.0 mL, 1.6 M) with vigorous stirring. A solution of Compound II-19 in dry THF (12 mg Compound II-19, 1 mL THF) was added slowly to the clear dialkylcuprate solution and the resulting mixture stirred at -78°C for 1 hr. The reaction was quenched by washing the THF solution through a plug of silica gel (1 cm diameter by 2 cm length) along with further washing using a solution of 50% EtOAc / 50% hexanes (50 mL). The combined silica plug washes were dried *in vacuo* and subjected to further C18 HPLC purification in 2 injections (ACE 5 μm C18-HL, 150 mm X 21 mm ID) using an isocratic solvent gradient consisting of 35% acetonitrile/65% H_2O . Compound II-30 eluted under these conditions at 23.5 minutes and yielded 2.4 mg material (27% isolated yield) at 90.8% purity as measured by analytical HPLC. An alternative normal phase purification method can be utilized using Phenomenex Luna 10 μm Silica column (25cm x 21.2 mm ID) with a solvent gradient consisting of 100% hexanes/ethyl acetate to 0% hexanes over 20 minutes. Compound II-30 eluted under these conditions at 16.5 minutes and yielded 3.0 mg material (41% isolated yield) at 97.1% purity as measured by analytical HPLC.

[0410] Compound II-30: UV (Acetonitrile/ H_2O) 225 (sh), ESMS, m/z 294.1 (M+H); HRMS (ESI), m/z 294.1696 [M+H] $^+$, $\Delta_{\text{calc}} = -3.2$ ppm, $\text{C}_{16}\text{H}_{24}\text{NO}_4$.



[0411] Compound II-30 can also be obtained by saline fermentation of strain CNB476. In one example, CNB476 was transferred to 500-mL flasks containing 100 mL production medium consisting of the following per liter of deionized water: starch, 10 g; yeast extract, 4 g; Hy-Soy, 4 g; ferric sulfate, 40 mg; potassium bromide, 100 mg; calcium carbonate, 1 g; and synthetic sea salt, 30 g. The production cultures were incubated at 28°C and 250 rpm for 1 day. Approximately 2 g of sterile Amberlite XAD-7 resin was added to the production cultures. The production cultures were further incubated for 5 days. The resin was recovered from the broth and extracted with ethyl acetate. The extract was dried *in vacuo*. The dried extract (8 g) was then processed for the recovery of Compound II-30.

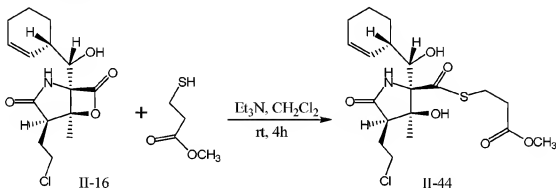
[0412] The crude extract was processed by flash chromatography using a Biotage Flash system. The flash chromatography was developed by the following step gradient: i) Hexanes (1L); ii) 10% EtOAc in hexanes (1L); iii) 20% EtOAc in hexanes, first elution (1L); iv) 20% EtOAc in hexanes, second elution (1L); v) 20% EtOAc in hexanes, third elution (1L); vi) 25% EtOAc in hexanes (1L); vii) 50% EtOAc in hexanes (1L); viii) EtOAc (1L). Fractions containing Compound II-30 was further purified by normal phase HPLC using an isocratic solvent system of 24% EtOAc/hexanes followed by a 100% EtOAc. Compound II-30 eluted 22 minutes into the isocratic portion of the run.

[0413] Fractions enriched in Compound II-30 were further processed by normal phase HPLC using a 27 minute linear gradient from 15% hexanes/85% EtOAc to 100% EtOAc. Compound II-30 eluted after 15 min.

EXAMPLE 24

SYNTHESIS OF THE COMPOUND OF FORMULAE II-44 FROM II-16

[0414] The compound of Formula II-16 (30 mg, 0.096 mmol) was dissolved in CH₂Cl₂ (9 mL) in a scintillation vial (20 mL) to which triethylamine (40 μ L, 0.29 mmol), methyl-3-mercapto propionate (thiol, 250 μ L) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 4 hours. The solvent was evaporated from the reaction mixture to yield a mixture of compound of Formulae II-44, which was separated by reversed phase HPLC using Ace 5 μ m C18 column (150 mm x 22 mm ID) with a solvent gradient of 35% to 90% H₂O/acetonitrile over 17 min, 90 to 100% acetonitrile over 1 min, holding at 100% acetonitrile for 1 min, at a flow rate of 14.5 mL/min. Diode array detector was used to monitor the purification process. Compound II-44 (20 mg) eluted at 11.68 min as a pure compound. Compound II-44: UV (Acetonitrile/H₂O) λ_{max} 240 (sh) nm; ESMS m/z 434.0 (M+H)⁺ & 456.0 (M+Na)⁺.



EXAMPLE 25

OXIDATION OF SECONDARY HYDROXYL GROUP IN COMPOUNDS OF

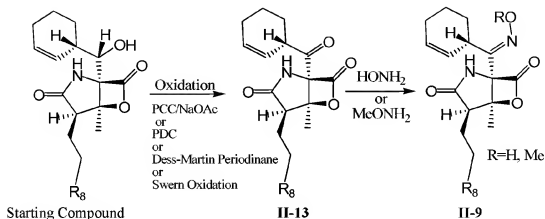
FORMULAE II-16, II-17 AND II-18

AND REACTION WITH HYDROXY OR METHOXY AMINES

[0415] Any of the compounds of Formulae II-16, II-17 and II-18 can be used as the starting compound. The secondary hydroxyl group in the starting compound is oxidized using either of the following reagents: pyridinium dichromate (PDC), pyridinium chlorochromate (PCC), Dess-Martin periodinane or oxalyl chloride (Swern oxidation) (Ref:

Organic Syntheses, collective volumes I-VIII). Preferably, Dess-Martin periodinane can be used as a reagent for this reaction. (Ref: Fenteany G. *et al.* Science, **1995**, 268, 726-73). The resulting keto compound is treated with hydroxylamine or methoxy amine to generate oximes.

Examples:

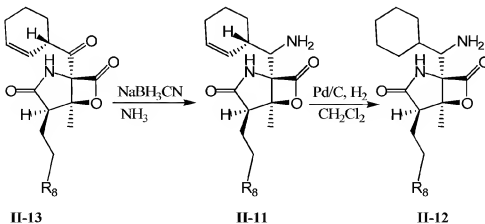


EXAMPLE 26

REDUCTIVE AMINATION OF KETO-DERIVATIVE

[0416] The keto derivatives, for example Formula II-8 and II-13, are treated with sodium cyanoborohydride (NaBH_3CN) in the presence of various bases to yield amine derivatives of the starting compounds which are subsequently hydrogenated with 10% Pd/C, H_2 to reduce the double bond in the cyclohexene ring.

Example:

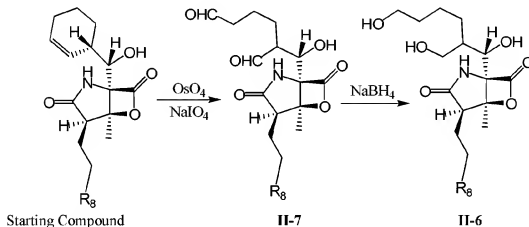


EXAMPLE 27

CYCLOHEXENE RING OPENING

[0417] Any compound of Formulae II-16, II-17 and II-18 can be used as a starting compound. The Starting Compounds can be protected, for example, at the alcohol and/or at the lactam nitrogen positions, and treated with OsO_4 and NaIO_4 in $\text{THF-H}_2\text{O}$ solution to yield dial derivatives which are reduced to the alcohol with NaBH_4 . The protecting groups can be removed at the appropriate stage of the reaction sequence to produce II-7 or II-6.

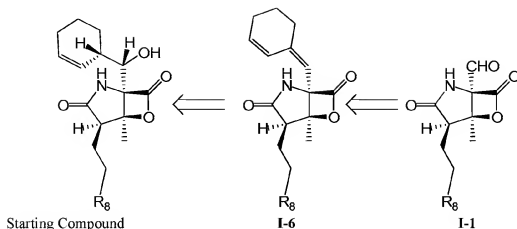
Example:



EXAMPLE 28

DEHYDRATION OF ALCOHOL FOLLOWED BY ALDEHYDE FORMATION AT LACTONE-LACTAM RING JUNCTION

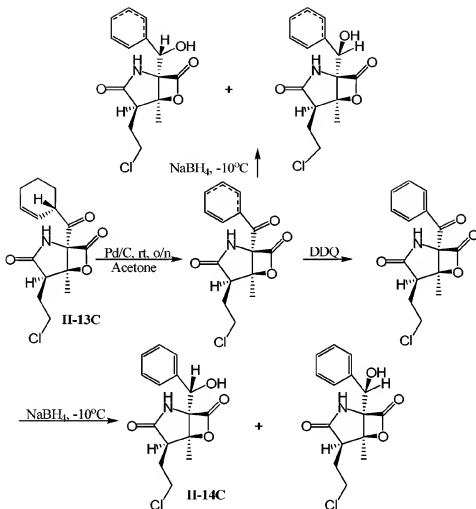
[0418] A starting compound of any of Formulae II-16, II-17 or II-18 is dehydrated, for example, by treatment with mesylchloride in the presence of base, or, for example, by treatment with Burgess reagent or other dehydrating agents. The resulting dehydrated compound is treated with OsO_4 , followed by NaIO_4 , or alternatively by ozonolysis, to yield an aldehyde group at the lactone-lactam ring junction.



EXAMPLE 29

OXIDATION OF THE CYCLOHEXENE RING TO PRODUCE CYCLOHEXADIENES
OR A PHENYL RING

[0419] A Starting Compound, such as the ketone of Formula II-13C, is treated with Pd/C to produce a cyclohexadiene derivative. The new double bond can be at any position of the cyclohexene ring. The ketone can be reduced, for example, with sodium borohydride, to obtain the corresponding secondary alcohol(s). Alternatively, the cyclohexadiene derivative can be further treated, for example with DDQ, to aromatize the ring to a phenyl group. Similarly, the ketone can be reduced, for example, with sodium borohydride, to obtain the corresponding secondary alcohol(s).

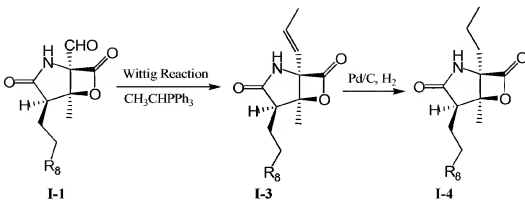


EXAMPLE 30

VARIOUS REACTIONS ON ALDEHYDE DERIVATIVES I-1

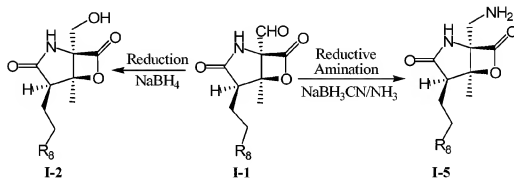
[0420] Wittig reactions are performed on the aldehyde group using various phosphorus ylides [e.g., (triphenylphosphoranylidene)ethane] to yield an olefin. The double bond in the side chain is reduced by catalytic hydrogenation.

Example:



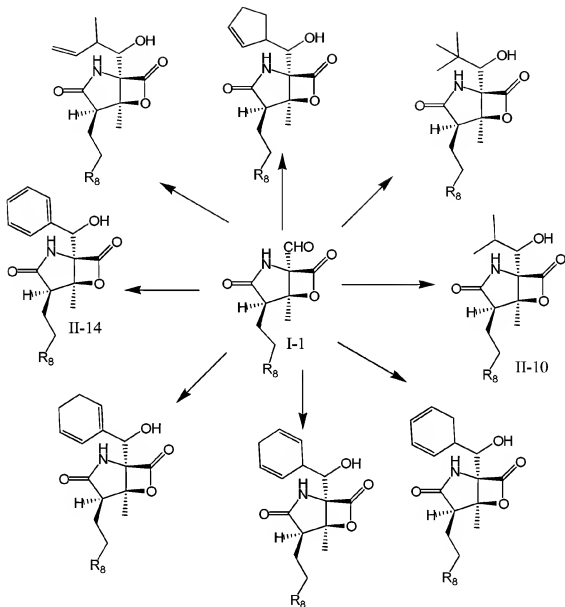
[0421] Reductive amination is performed on the aldehyde group using various bases (eg. NH_3) and sodium cyanoborohydride to yield amine derivatives. Alternatively, the aldehyde is reduced with NaBH_4 to form alcohols in the side chain.

Example:



[0422] Organometallic addition reactions to the aldehyde carbonyl can be performed to yield various substituted secondary alcohols.

Examples:

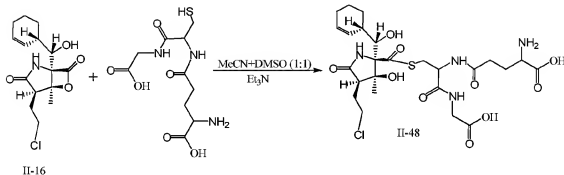


EXAMPLE 31

SYNTHESIS OF THE COMPOUND OF FORMULAE II-48 FROM II-16

[0423] The compound of Formula II-16 (15 mg, 0.048 mmol) was dissolved in 1:1 ratio of acetonitrile/DMSO (8 mL) in a scintillation vial (20 mL) to which triethylamine (40 μ L, 0.29 mmol), Glutathione (44.2 mg, 0.144 mmol) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 3 hours. The solvent was

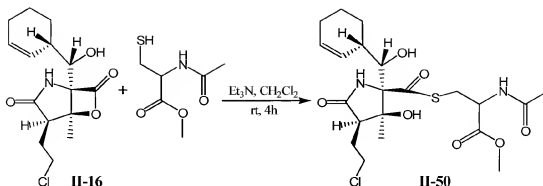
evaporated from the reaction mixture to yield the compound of Formula II-48, which was purified by reversed phase HPLC using Ace 5 μ m C18 column (150 mm x 22 mm ID) with a solvent gradient of 10% to 70% H₂O/acetonitrile over 15 min, 70 to 100% acetonitrile over 5 min, holding at 100% acetonitrile for 4 min, at a flow rate of 14.5 mL/min. Diode array detector was used to monitor the purification process. Compound II-48 (10 mg) eluted as a pure compound at 8.255 min. Compound II-48: UV (Acetonitrile/H₂O) λ_{max} 235 (sh) nm; ESMS m/z 621.0 (M+H)⁺.



EXAMPLE 32

SYNTHESIS OF THE COMPOUND OF FORMULA II-50 FROM II-16

[0424] The compound of Formula II-16 (10 mg, 0.032 mmol) was dissolved in CH₂Cl₂ (9 mL) in scintillation vial (20 mL) to which triethylamine (26.5 μ L, 0.192 mmol), N-Acetyl-L-Cysteine methyl ester (17 mg, 0.096 mmol) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 4 hours. The solvent was evaporated from the reaction mixture to yield the mixture of compound of Formulae II-50, which was further purified by normal phase HPLC using Phenomenex Luna 10 μ m Silica column (25 cm x 21.2 mm ID) with a solvent gradient of 10% to 100% Hexane/EtOAc over 24 min, holding at 100% EtOAc for 3 min, at a flow rate of 14.5 mL/min. ELSD was used to monitor the purification process. Compound II-50 (2 mg) was eluted at 10.39 min as a pure compound. Compound II-50: UV (Acetonitrile/H₂O) λ_{max} 230 (sh) nm; ESMS m/z 491.1 (M+H)⁺ & 513.0 (M+Na)⁺.



EXAMPLE 33

FORMULATION TO BE ADMINISTERED ORALLY OR THE LIKE

[0425] A mixture obtained by thoroughly blending 1 g of a compound obtained and purified by the method of the embodiment, 98 g of lactose and 1 g of hydroxypropyl cellulose is formed into granules by any conventional method. The granules are thoroughly dried and sifted to obtain a granule preparation suitable for packaging in bottles or by heat sealing. The resultant granule preparations are orally administered at between approximately 100 mL/day to approximately 1000 mL/day, depending on the symptoms, as deemed appropriate by those of ordinary skill in the art of treating cancerous tumors in humans.

EXAMPLE 34

FORMULATION TO BE ADMINISTERED ORALLY OR THE LIKE

[0426] A mixture obtained by thoroughly blending 1 g of a compound obtained and purified by the method of the embodiment, 98 g of lactose and 1 g of hydroxypropyl cellulose is formed into granules by any conventional method. The granules are thoroughly dried and sifted to obtain a granule preparation suitable for packaging in bottles or by heat sealing. The resultant granule preparations are orally administered at between approximately 100 mL/day to approximately 1000 mL/day, depending on the symptoms, as deemed appropriate by those of ordinary skill in the art of treating cancerous tumors in humans.

EXAMPLE 35

INDUCTION OF APOPTOSIS ALONE AND IN COMBINATION WITH HDAC
INHIBITORSMATERIALS AND METHODSCells

[0427] Jurkat, K562, ML-1 and 12.1 (FADD deficient Jurkat) human leukemia cell lines were purchased from American Type Culture Collection (Rockville, MD). Caspase-8 deficient Jurkat cells, 19.2, were obtained from University of Texas, M. D. Anderson Cancer Center, Houston, TX. All cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), L-glutamate and penicillin/streptomycin (Sigma, St. Louis, MO). Cells were maintained at 37°C with 5% CO₂. Peripheral blood were obtained from a Philadelphia positive (Ph+) ALL patient. Mononuclear cells were isolated using double density Ficoll-Hypaque gradients composed of Histopaque 1077 and 1119 (Sigma, St. Louis, MO) as previously described (*see* Chandra J, Hackbarth J, Le S, et al. Involvement of reactive oxygen species in adaphostin-induced cytotoxicity in human leukemia cells. Blood. 2003; 102:4512-4519).

Reagents

[0428] Salinosporamide A was obtained from Nereus Pharmaceuticals, bortezomib was obtained from the M.D. Anderson Cancer Center pharmacy. HDACi's, (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275) and valproic acid (VPA), were obtained from Calbiochem and Sigma (San Diego, CA and St. Louis, MO). Fluorogenic substrates, suc-LLVY-amc and z-LLE-amc, were from AG Scientific, Inc. (San Diego, CA) and boc-LLR-amc from Bachem (King of Prussia, PA). NAC and staurosporine, were purchased from Sigma (St. Louis, MO). The dyes 6-carboxy-2',7' - dichlorodihydrofluorescein (H2DCF-DA), dihydroethidium (HET) and tetramethylrhodamine ethyl ester (TMRE) were obtained from Molecular Probes (Eugene, OR). Antibodies were obtained from: Caspase-8, caspase-9, caspase-3, FADD, and Bid (Cell Signaling, Beverly, MA), CH-11 (MBL International, Woburn, MA), p27 (Transduction Laboratories, San Diego, CA), cytochrome c (BD PharMingen, San Diego, CA), and actin (Sigma, St. Louis, MO). The caspase inhibitors zVAD-fmk, IETD-fmk and LEHD-fmk were purchased from

Calbiochem (San Diego, CA). The caspase-3 substrate, DEVD-amc, was obtained from Biomol International, LP (Plymouth Meeting, PA). Annexin V-FITC was purchased from BD Bioscience (Franklin Lakes, NJ).

20S Proteasome Activity Assay

[0429] The chymotrypsin-like, trypsin-like and caspase-like activity of the 20S proteasome of leukemia cells can be determined by measurement of fluorescence generated from the cleavage of the fluorogenic substrates suc-LLVY-amc, boc LRR-amc and z-LLE-amc respectively (*see* Lightcap ES, McCormack TA, Pi en CS, Chau V, Adams J, Elliott PJ. Proteasome inhibition measurements: clinical application. Clin Chem. 2000;46:673-683). Cells were incubated for 1 h in the presence of diluent or 1 μ M Salinosporamide A, washed with phosphate buffered saline (PBS), and resuspended in 300 μ L of a solution containing 20 mM Tris, pH 7.5, 0.1 mM EOTA, pH 8.0, 20% glycerol, 0.05% Nonidet-P40, 1 mM 2-13 mercaptoethanol, 1 mM ATP and lysed by freezing and thawing three times on dry ice. After centrifugation, supernatants were combined with substrate buffer (50 mM HEPES, pH 7.5, 5 mM EGTA pH 7) and the specific fluorogenic substrate in a 96-well plate and analyzed on a spectrofluorometer (SpectraMax Gemini EM, Molecular Devices, Sunnyvale, CA) using an excitation of 380 nm and an emission of 460 nm.

Western Blotting

[0430] Cells (5×10^6) were incubated with indicated concentrations of Salinosporamide A and washed with PBS followed by lysing as previously described (*see* Chandra J, Niemer I, Gilbreath J, et al. Proteasome inhibitors induce apoptosis in glucocorticoid-resistant chronic lymphocytic leukemic lymphocytes. Blood. 1998;92:4220-4229).

[0431] Aliquots of protein lysates (50 μ g) were run on SOS-polyacrylamide gels. Protein was transferred to nitrocellulose membranes and blocked either for 1 h at room temperature or overnight at 4°C with 5% non-fat dry milk. Membranes were incubated with a 1:1000 dilution of primary antibodies in 5% milk/Tris-buffered saline with 0.05% Tween-20 (TBST), followed by corresponding secondary antibodies (1:1000 dilution with 5% milk/TBST). Bound antibodies were detected using enhanced chemiluminescence (ECL plus

western blotting detection system, Amersham Biosciences UK limited, Little Chalfont Buckinghamshire, England).

Assessment of DNA Fragmentation

[0432] Apoptosis was assessed by propidium iodide (PI) staining followed by fluorescence-activated cell sorting (FACS) analysis as described previously (see Chandra J, Hackbarth J, Le S, et al. Involvement of reactive oxygen species in adaphostin-induced cytotoxicity in human leukemia cells. Blood. 2003; 1 02:4512-4519). Following incubation with different doses of Salinosporamide A for 24 h, cells were pelleted by centrifugation and resuspended with PBS containing 50 µg/mL PI, 0.1 % Triton-X-1 00, and 0.1 % sodium citrate. Samples were stored at 4°C for 24 h and vortexed before analysis on the FL-3 channel of a flow cytometer (FACSCalibur; Becton Dickinson; Franklin lakes, NJ). Data were analyzed using Cell Quest Software (BD Bioscience, Franklin lakes, NJ).

Annexin V staining

[0433] Externalization of phosphatidylserine (PS) was measured by Annexin V-FITC staining according to the manufacturer's protocol. Briefly, 1x10⁶ cells were treated with indicated doses of Salinosporamide A for 6 h, washed twice in cold PBS and resuspended in 1X binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM CaCl₂) and incubated for 30 min in the dark at room temperature with 5 µl Annexin V-FITC and 10 µl of 50 µg/mL PI. Samples were analyzed by flow cytometry.

In Vivo studies

[0434] Eight to 12 week old male CB.17/SCID mice were purchased from Taconic Farms (Hudson, NY). SCID mice were injected Lv. (tail vein) with 2 x 10⁷ ML-1 cells in 0.2 mL of PBS. Viability of injected cells was >90% as assessed by trypan blue exclusion. Mice were separated into control and treatment groups (n = 6/group) and treatment with Salinosporamide A (0.15 mg/kg) or vehicle alone (1 % DMSO in PBS) was given intraperitoneally (I.p.) twice a week for 5 weeks. Blood samples were collected on days 17, 21, 23, 32 and 35. To avoid tail necrosis, the same mouse was not bled at every time point, accounting for nine bleeds from each group (control versus treated) over the course of 35 days. Complete cell blood counts were performed using a minimum of 50 µL of peripheral blood using a Cell-Dyn (Abbott Diagnostics, Abbott Park, IL) counter. Post hoc power

analysis indicated that with 9 observations per group (control or Salinosporamide A), 26% power was had to see a statistically significant ($p < 0.05$) difference in slopes of the regression lines for WBC count (tumor burden) over time. With 36 observations per group 80% power have had to been obtained to see a statistically significant difference between the slopes of the regression lines for WBC count over time.

Transient Transfection

[0435] Full length FADD EGFP-N1 plasmid 30, was transfected into FADD deficient 12.1 cells using Nucleofector Kit V (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's protocol. After 24 h, the brightest 25%GFP positive cells of the total population were sorted by FACS. These GFP positive cells were treated with 200 nM Salinosporamide A for 6 h and analyzed for caspase-3 activity.

Caspase-3 Activity Assays

[0436] Cells were pelleted, resuspended in 150 μ L PBS, and lysed by freezing and thawing. Fifty μ L was loaded in triplicate on a 96-well plate. To each well, 150 μ L of 50 μ M DEVD-amc in DEVD Buffer (10% sucrose, 0.001 % IGEPAL, 0.1 % CHAPS, 5 mM HEPES, pH 7.25) was added. Release of fluorescence (amc) was measured using a spectrofluorometer using an excitation of 355 nm and an emission of 460 nm.

Detection of Intracellular Peroxides and Superoxide

[0437] Measurements of intracellular ROS were determined by using cell permeable dyes as previously described 31. Pelleted cells were resuspended in 1 mL of RPMI medium containing either 10 μ M CM-H2DCF-DA or 10 μ M HEt (measuring intracellular peroxide and superoxide levels, respectively), and incubated at 37°C for 30 min in the dark. Fluorescence intensity was read by flow cytometry on the FL-1 (DCF) or FL-3 (HEt) channel.

Measurement of Changes in Mitochondrial Membrane Potential

[0438] Cells were pelleted, washed with PBS, and incubated with 25 nM TMRE in 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$, pH 7.4 in a volume of 1 mL for 30 min at 37°C in the dark. Cells were washed in PBS and fluorescence intensity was analyzed on the FL-2 channel of a flow cytometer.

Statistical Analyses

[0439] Values represent the mean S.D. from three separate studies performed in triplicate. Differences in groups were assessed by using paired Student's t-test and were considered statistically significant at $p < 0.05$. For the experiments combining proteasome inhibitors and HDACi, synergism was determined using isobologram analysis based on the Chou and Talalay method with Calcsyn (Biosoft, Ferguson, MO) software program 32. A combination index (CI) value < 1.0 indicates synergism: from 0.1-0.3 indicates strong synergism, and from 0.3-0.7 synergism. A CI=1.0 indicates additive effects. In vivo data were analyzed with linear regression to estimate the rate of change (i.e., the slope of the regression line) of the WBC count over time for the mice treated with diluent or Salinosporamide A.

RESULTS

[0440] Salinosporamide A inhibits the 20S proteolytic activities in leukemia cells. To measure Salinosporamide A's effects on the proteolytic activities of the 20S proteasome in leukemia cells, cell lines representative of CML, ALL and AML (K562, Jurkat and ML-1 respectively) were used. Cells were incubated with 1 μM Salinosporamide A for 1 h and the chymotrypsin-like, caspase-like and trypsin-like activities were measured using distinct fluorogenic peptides. Figures 1A and 1B show that 1 μM Salinosporamide A inhibited the chymotrypsin-like and caspase-like activity by greater than 90% compared to diluent in the three leukemia cells lines, whereas the trypsin-like activity was inhibited to a lesser extent (Figure 1C). The accumulation of p27 (Figure 1D) and p21 (data not shown), both known proteasome protein substrates, in cells treated with Salinosporamide A served as functional confirmation of proteasome inhibition.

[0441] The effects of Salinosporamide A and bortezomib on the chymotrypsin-like and caspase-like activities were compared. At the 1 μM dose, Salinosporamide A inhibited both the the chymotrypsin-like and caspase-like activities more effectively than bortezomib ($p = 0.005$ and $p = 0.019$) in Jurkat cells (Figure 1E). To further characterize the ability of Salinosporamide A (NPI-0052) to inhibit proteasome activities in comparison to bortezomib, dose dependent effects of the two inhibitors were assessed (Figure 1E). At the 200 nM dose, Salinosporamide A was more effective than bortezomib at inhibiting the chymotrypsin-like, caspase-like and trypsin-like activities in Jurkat cells.

[0442] Salinosporamide A induces apoptotic cell death *in vitro* and *in vivo*. The cytotoxic effects of Salinosporamide A in K562, Jurkat and ML-1 cells were examined next. These cell lines were exposed to 1 nM - 1 μ M Salinosporamide A for 24 h. Salinosporamide A induced DNA fragmentation, which peaked at 200 nM, in a dose dependent manner as measured by PI staining in the three leukemia cell lines (Figure 2A). An increase of Annexin V positive and PI negative cells were detected after 6 h of treatment with various doses of Salinosporamide A (19.2% for 10 nM, 28.9% for 50 nM and 28.7% for 200 nM) (Figure 2A inset). Cells were also collected after 1 h exposure with Salinosporamide A and analyzed for inhibition of chymotrypsin-like activity using these same doses. Salinosporamide A doses that induced apoptosis also effectively inhibited the chymotrypsin-like activity in Jurkat cells (Figure 2B). Proteasome inhibition by Salinosporamide A triggered apoptosis in a time dependent fashion, with maximum DNA fragmentation occurring at 24 h (Figure 2C). At a lower dose (10 nM), Salinosporamide A achieved significant proteasome inhibition but did not cause DNA fragmentation. Analysis of phosphatidylserine exposure, an early apoptotic event, at the 10 nM dose, however revealed that 19.2% of cells were Annexin V positive. These findings indicates that higher doses of Salinosporamide A are required to induce apoptosis than those needed to inhibit proteasome activity.

[0443] The ability of Salinosporamide A to exert effects *in vivo* was examined by reconstituting SCID mice with ML-1 cells. Biweekly administration of 0.15 mg/kg Salinosporamide A decreased tumor burden as evidenced by lower total white blood cell counts (WBC; Figure 2D) over the course of five weeks. Linear regression modeling of WBC over time found a difference in the y-intercepts of the regression lines ($p = 0.06$) comparing mice administered Salinosporamide A ($n = 6$ with 9 observations) versus diluent ($n = 6$ with 9 observations). It is worth noting that the slope of the regression line for the Salinosporamide A group was negative (-0.0244) and the slope of the regression line for the control group was positive (0.0317), and that the two lines intersect on the first day of treatment (day 2).

[0444] Since DNA fragmentation is a consequence of caspase-3 activation, caspase-3 activity was examined in response to Salinosporamide A (Figure 3A). Treatment with anti-Fas antibody (CH-11) served as a positive control for caspase-3 activation. As

shown in Figure 3A, 200 nM Salinosporamide A increased caspase-3 activity by 4-fold as compared to control. Inhibition of caspases by a pan-caspase inhibitor, zVAD-fmk, attenuated the Salinosporamide A induced caspase-3 activity. Furthermore, 10 μ M IETD-fmk, a caspase-8 inhibitor, abrogated caspase-3 activity by Salinosporamide A ($p < 0.001$), whereas an equimolar dose of caspase-9 inhibitor, LEHD-fmk, did not. The combination of LEHD-fmk and staurosporine was used as a positive control to demonstrate the effectiveness of the caspase-9 inhibitor (Figure 3A). Salinosporamide A induced caspase-3 activity in a time dependent manner, beginning at 4 h and plateauing at 8 h (Figure 3B). Detection of 19 kD and 17 kD cleaved products by Western blot analysis (Figure 3B inset) further confirmed caspase-3 activation by Salinosporamide A.

[0445] Caspase-8 activation by Salinosporamide A. To verify a role for caspase-8 activation as an early event in Salinosporamide A-induced cell death, cleavage of caspase-8 in Jurkat cells was measured (Figure 3C) over a 6 h time period by immunoblotting. Exposure of Jurkat cells to 200 nM Salinosporamide A caused activation of caspase-8 starting at 2 h indicated by the appearance of the 43 kD and 41 kD cleavage fragments.

[0446] The relative contributions of caspase-8 and caspase-9 on Salinosporamide A induced apoptosis were assessed using peptide inhibitors. Jurkat cells were treated with 200 nM Salinosporamide A alone or in combination with caspase-8 and caspase-9 inhibitors for 24 h and DNA fragmentation was assessed. When pretreated with a caspase-8 inhibitor, Jurkat cells were protected against Salinosporamide A induced apoptosis in a statistically significant manner ($p < 0.001$), whereas a caspase-9 inhibitor did not confer protection (Figure 3D).

[0447] Bid is a pro-apoptotic BH-3 domain containing member of the Bcl-2 family and is a substrate for caspase-8 36. Exposure to 1 μ M Salinosporamide A for 8 h induced the cleavage of Bid (Figure 3E), generating a 15 kDa fragment, tBid, which can translocate to mitochondria. Thus, while not being bound by any particular theory, Salinosporamide A is likely exerting its cytotoxic effects through a caspase-8-tBid-mitochondria-dependent pathway.

[0448] Mitochondrial injury by Salinosporamide A was evaluated by detecting drops in mitochondrial membrane potential ($\Delta\psi_m$). Reduction in TMRE fluorescence

(indicative of loss of $\Delta\Psi_m$) by 56.7% was seen in Jurkat cells treated with 1 μM Salinosporamide A for 6h (Figure 3F). This drop in potential was not abrogated by an antioxidant, NAC. Staurosporine, a positive control, caused a 96.8% reduction in $\Delta\Psi_m$. These results support a model in which Salinosporamide A causes caspase-8 activation, leading to Bid cleavage and mitochondrial perturbations.

[0449] Loss of mitochondrial membrane potential generally precedes cytochrome c release from mitochondria which leads to caspase-9 activation in the cytosol 37. Figure 3G shows the presence of cytochrome c in cytosol after 4 h of Salinosporamide A treatment but not in diluent treated cells. Accordingly, levels of pro-caspase-9 were decreased in Salinosporamide A treated cells, indicating activation of the zymogen (Figure 3G).

[0450] Several reports indicate that proteasome inhibition causes increased ROS levels. To determine if ROS levels are heightened by Salinosporamide A, Jurkat cells were treated with 200 nM Salinosporamide A and stained with H₂Et or DCF. Monitoring ROS over time revealed strong increases in intracellular superoxide and hydrogen peroxide after 14 h of exposure (Figure 3H).

[0451] Requirement for caspase-8 in Salinosporamide A induced apoptosis in leukemia cells. In order to address if caspase-8 is required for Salinosporamide A induced apoptosis, Jurkat cells lacking caspase-8 (I9.2) were utilized and cells lacking the caspase-8 adaptor molecule FADD (I2.1). The absence of caspase-8 and FADD was confirmed by western blotting (Figure 4A). Both of these cell lines showed significant inhibition of chymotrypsin-like proteasome activity after exposure to 1 μM Salinosporamide A (data not shown), indicating that caspase-8 and FADD are not required for Salinosporamide A to inhibit the proteasome.

[0452] Caspase-3 activity was assessed after Salinosporamide A exposure in I2.1 and I9.2 cells. Caspase-8 and FADD deficient cell lines moderately increased caspase-3 activity 3-fold, whereas wild type Jurkat cells exhibited a 6-fold increase as compared to control (Figure 4B). Thus, caspase-8 and FADD mediate Salinosporamide A-induced caspase-3 activity.

[0453] The specific requirement for FADD in Salinosporamide A-induced apoptosis was further tested by transfection of wildtype FADD-GFP into the FADD deficient

I2.1 cells and subsequent treatment with 200 nM Salinosporamide A (Figure 4C). In comparison to parental I2.1 cells, the FADD transfectants displayed higher levels of caspase-3 activity and the difference was statistically significant ($p < 0.05$). As a positive control, 200 ng/mL CH-11 was shown to induce DNA fragmentation in I2.1 FADD-GFP transfectants (53.27%) but not in the I2.1 cell line (4.99%) (Figure 4C inset).

[0454] To place caspase-8 activation in the context of other biochemical apoptotic events, alterations of $\Delta\Psi_m$ in parental, caspase-8 deficient and FADD deficient Jurkat cells were examined. Jurkat cells exposed to 200 nM Salinosporamide A displayed a loss of $\Delta\Psi_m$ as detected by TMRE staining and indicated by a shift of the histogram (Figure 4D). The caspase-8 and FADD deficient, I9.2 and I2.1, cells did not display a drop in $\Delta\Psi_m$ in the presence of Salinosporamide A. These data indicate that caspase-8 and FADD are required for Salinosporamide A to cause mitochondrial perturbations.

[0455] Requirement for Salinosporamide A induced ROS in cytotoxicity and proteasome inhibition. To test if increased levels of ROS by Salinosporamide A contribute to apoptosis, Jurkat cells were treated with 200 nM Salinosporamide A alone or in combination with the antioxidant, NAC. Figure 5A shows that an 8 h exposure to Salinosporamide A caused an increase in caspase-3 activity that was attenuated 3-fold in the presence of NAC. Superoxide levels generated by 100 nM Salinosporamide A were blunted in the presence of NAC (Figure 5A inset). Furthermore, the antioxidant conferred significant protection ($p < 0.001$) against Salinosporamide A induced apoptosis as measured by DNA fragmentation (Figure 5B). Similarly, in mononuclear cells isolated from a Ph+ ALL patient, NAC also protected against cell death by Salinosporamide A (Figure 5C).

[0456] Examination of proteasome activity in Jurkat cells revealed that protection by NAC was not due to interference with Salinosporamide A effects on proteasome function (Figure 5D). In addition, western blot analysis results demonstrate that NAC did not prevent caspase-8 or Bid cleavage in Jurkat cells treated with Salinosporamide A (Figure 5E). Interestingly, neither pan-caspase inhibitors nor specific caspase-8 inhibitors inhibited superoxide production by Salinosporamide A (Figure 5F). It was also observed that NAC does not protect from alterations to mitochondrial membrane potential (Figure 3F). These

results indicate that inhibition of the proteasome and increases in ROS by Salinosporamide A are independent of caspase-8 and Bid activation and mitochondrial perturbations.

[0457] Salinosporamide A interacts with HDACi to induce synergistic apoptosis. It was examined whether the HDACi, MS-275 and VPA, can be combined with Salinosporamide A to enhance apoptosis in leukemia cells. Jurkat cells were treated with increasing doses of MS-275 (1 - 5 μ M) or VPA (1 - 5 mM) and low doses of Salinosporamide A (10 nM or 5 nM) for 24 h. The combination of 2.5 μ M or 5 μ M MS-275 and 10 nM Salinosporamide A significantly increases DNA fragmentation ($p < 0.05$) when compared to cells exposed to a single agent (Figure 6A). Furthermore, cells treated with 10 nM Salinosporamide A and MS-275 had a higher percent increase in the subdiploid population (Figure 6C, $p < 0.05$) and displayed greater synergism ($CI = 0.27$ for 2.5 μ M MS-275 and $CI = 0.21$ for 5 μ M MS-275) compared with cells treated with 10 nM bortezomib and MS-275 ($CI = 0.48$ for 2.5 μ M MS-275 and $CI = 0.47$ for 5 μ M MS-275). To explore the dependence upon caspase-8 of this observed synergy, the comparison of parental Jurkat cells to caspase-8 deficient counterparts was undertaken (19.2). As predicted by the subdiploid data, caspase-3 activation was potentiated in parental Jurkat cells treated with the combination of Salinosporamide A and MS-275 (Figure 6B; $p < 0.001$). However, caspase-8 deficient cells did not display additive or synergistic effects. Combined treatment of Salinosporamide A and MS-275 resulted in an increase of intracellular superoxide levels (Figure 6B inset). Exposure of Jurkat cells to low doses of VPA (1 or 2.5 mM) and Salinosporamide A (5 nM) also displayed significantly enhanced apoptosis (Figure 6D, $p < 0.05$). As shown in Figure 6D (inset), treatment with 5 nM Salinosporamide A and 2.5 mM VPA resulted in a greater increase in the subdiploid population ($p < 0.05$) as compared with 5 nM bortezomib and VPA. Finally, combination index values indicated synergism between 5 nM Salinosporamide A and 2.5 mM VPA ($CI = 0.70$), while 5 nM bortezomib and 2.5 mM VPA displayed additive effects ($CI = 1.06$). These findings demonstrate for the first time that combination of HDACi, (either MS-275 or VPA), with low doses of Salinosporamide A results in synergistic induction of apoptosis and these effects are more potent than those seen when bortezomib is combined with the same agents.

DISCUSSION

[0458] Recent studies have described the effects of Salinosporamide A in myeloma cell lines and animal models, and in primary chronic lymphocytic leukemia (CLL) cells. The current study discloses Salinosporamide A's effects on proteasome activity and the apoptotic machinery in other hematological malignancies, with a focus on ALL and AML model systems. In the current study, it is shown that Salinosporamide A inhibits all three activities associated with the 20S proteasome in leukemia cells (Figure 1) and induces apoptosis in a variety of leukemia cells. Cells representative of ALL, CML, AML, as well as mononuclear cells from a Ph+ ALL patient are sensitive to Salinosporamide A (Figure 2A and 5C). In vivo administration of Salinosporamide A also decreases tumor burden in leukemia bearing mice (Figure 2E). These data demonstrate a spectrum of activity that extends to numerous hematological malignancies.

[0459] Several unique features of Salinosporamide A hint at the potential for efficacy in diseases where bortezomib has been less successful. Firstly, Salinosporamide A inhibits the 20S proteolytic activities in leukemic cells to different degrees, blocking the chymotrypsin-like and caspase-like activities more effectively than the trypsin-like activity (Figure 1A-C). In addition, Salinosporamide A was found to be more potent than bortezomib in inhibiting the rate-limiting activity of the proteasome (Figure 1E). It has been reported that proteasome activities are allosterically regulated and that inhibition of multiple sites of the proteasome is necessary to block significant protein degradation, Salinosporamide A's pattern of inhibition may impact proteasome function.

[0460] The mode of apoptosis induction by Salinosporamide A may also be influenced by this unique differential inhibition of proteasome activities. Caspase-8 inhibition appears to be critical for Salinosporamide A's cytotoxicity whereas caspase-9 inhibitors did not significantly protect against DNA fragmentation or caspase-3 activation (Figure 3A, D). Nevertheless, pro-enzyme caspase-9 disappearance is detected by western blot (Figure 3G) indicating activation of caspase-9 by Salinosporamide A. Since upstream caspases, caspase-8 in this case, can cause activation of other caspases, it is likely that the disappearance of pro-caspase-9 is via this mechanism. These results further suggest that caspase-8 is activating caspase-9 since experiments using caspase-8 inhibitors, or ALL cell lines lacking caspase-8m or FADD showed diminished mitochondrial perturbations, caspase-3 activity, and DNA

fragmentation (Figures 3A, 3D and 4B, 4D). These results place caspase-8 at the apex of the apoptotic cascade triggered by Salinosporamide A (Figure 7). Use of lymphocyte models with total caspase-8 and FADD deficiency, extends and confirms data from multiple myeloma cell lines transfected with dominant- negative caspase-8, caspase-9 or FADD constructs. Furthermore, these studies show Bid cleavage and mitochondrial perturbations by Salinosporamide A in wildtype Jurkat cells, whereas caspase-8 and FADD deficient Jurkat cells do not undergo mitochondrial potential drops (Figure 4D). These results link Salinosporamide A to caspase-8: causing cleavage of Bid, loss of mitochondrial membrane potential, cytochrome c release, caspase-9 activation, caspase-3 activation and DNA fragmentation (Figure 7).

[0461] The antioxidant, NAC, conferred protection against Salinosporamide A induced caspase-3 activity and apoptosis (Figure 5A, 5B). Furthermore, increased peroxide and superoxide levels are detectable after exposure to Salinosporamide A (Figure 3H). Since caspase-8 activation proceeds in the presence of NAC (Figure 5E), and caspase-8 inhibitors do not affect Salinosporamide A's ability to raise intracellular superoxide levels (Figure 5F), these data place ROS alterations in a parallel pathway. Consistent with this model, NAC does not alter proteasome activity (Figure 5D). Several structurally dissimilar proteasome inhibitors are reported to cause elevated levels of intracellular superoxide and intracellular peroxides. The source of this oxidant production is of interest since inhibition of ROS by NAC prevents cytotoxicity in numerous models.

[0462] These results show for the first time that HDACi and low-doses of novel proteasome inhibitor, Salinosporamide A, synergistically induce apoptosis in leukemic cells. Synergistic interactions in leukemia cells exposed to low doses of Salinosporamide A and MS-275 or VPA (Figure 6) were observed. Whereas in cells treated with bortezomib and HOACi, less synergistic and additive effects were observed with MS-275 and VPA, respectively (Figure 6C and 6D inset). The mechanism by which Salinosporamide A synergizes with HDACi still remains to be resolved. However, HDACi have been shown to raise intracellular ROS levels and these results indicate that the combination of Salinosporamide A and MS-275 cause a further increase in superoxide than seen with either agent alone (Figure 6B inset). Thus it is conceivable that this greater oxidative challenge may

contribute to the synergistic effects. These results also implicate caspase-8 in the synergistic apoptosis induced by Salinosporamide A and MS-275 (Figure 6B). This data reveals the potential of administering Salinosporamide A at low doses (nontoxic) with HDACi, such as vorinostat (suberoylanilide hydroxamic acid (SAHA) or Zolinza®), for clinical benefit.

[0463] Taken together, this data suggests that Salinosporamide A is a potent proteasome inhibitor with potential therapeutic value in several hematologic malignancies, such as refractory solid tumors and lymphoma.

EXAMPLE 36

SYNERGISTIC ACTIVITY WITH HISTONE DEACETYLASE INHIBITORS

[0464] For the experiments using Salinosporamide A (NPI-0052) and HDAC inhibitor combinations, synergism was determined using isobologram analysis based on the method by Chou and Talalay (Adv Enzyme Regul 1984, 22: pages 27-55). A combination index (CI) value higher than 1.0 indicates synergism and a CI from 0.1-0.3 indicates strong synergism. A CI of 1.0 indicates additive effects and a CI of >1.0 antagonism.

[0465] Salinosporamide A, and histone deacetylase inhibitors (HDACi) synergistically induce apoptosis in leukemia cell lines in a caspase-8 and oxidant dependent manner. Furthermore, these lethal effects with Salinosporamide A and HDACi were more potent than those obtained when HDACi were combined with the reversible proteasome inhibitor, bortezomib (Velcade). To determine the mechanisms by which Salinosporamide A and HDACi may be synergizing, inhibition of proteasome activity and histone acetylation was examined, the proximal targets of these compounds. Increased acetylation of histone-3 was detected by western blot in Jurkat (T-cell ALL) cells treated with both Salinosporamide A and MS-275 compared to cells exposed to either single agent. Interestingly, these results indicate that the proteasome inhibitor is also eliciting classical chromatin associated epigenetic alterations. In addition, real-time PCR revealed that three structurally different HDACi, MS-275, SAHA (Zolinza/vorinostat) and valproic acid (VPA), decreased expression of the proteasomal $\beta 5$ subunit, which contains the rate limiting activity of the proteasome: the chymotrypsin-like (CT-L) activity. This reduction of $\beta 5$ expression affected proteasome function since we detect a decrease in CT-L activity after 12 h with 5 μ M MS-275 compared

to cells treated with diluent ($P < .01$). Further inhibition of CT-L activity was achieved with the combination of 5 μM MS-275 and 0.5 nM Salinosporamide A compared to either treatment alone ($P < .005$). This effect was reversed by N-acetyl-cysteine (NAC), an antioxidant. Ex vivo combination treatment of mononuclear cells from an AML patient with 5 nM Salinosporamide A and HDACi, MS-275 (1-5 μM) or vorinostat (suberoylanilide hydroxamic acid (SAHA)) (100-500 nM), resulted in an increase of the subdiploid population. Analysis of a range of doses using the Chou and Talalay method determined Salinosporamide A and HDACi treatment displayed greater synergism ($\text{CI} = 0.241$ for 5 μM MS-275 and $\text{CI} = 0.248$ for 500 nM vorinostat (suberoylanilide hydroxamic acid (SAHA))) compared to bortezomib ($\text{CI} = 0.401$ for 5 μM MS-275 and $\text{CI} = 0.701$ for 500 nM vorinostat (suberoylanilide hydroxamic acid (SAHA))). These data, together with the effects of HDACi on $\beta 5$ subunit expression and proteasome activity, and Salinosporamide A on acetylation reinforces the potential clinical utility of combining these two compounds. Overall, our results suggest that overlapping activities by Salinosporamide A and MS-275 are contributing, along with caspase-8 activation and oxidative stress, to their synergistic cytotoxic effects in leukemia cells.

EXAMPLE 37

EFFECT OF SALINOSPORAMIDE A IN COMBINATION WITH HDAC INHIBITORS ON HEMATOLOGIC MALIGNANCIES

[0466] Salinosporamide A interacts with HDACi (MS-275) to induce synergistic apoptosis. It was examined whether the HDACi, MS-275, can be combined with Salinosporamide A to enhance apoptosis in acute myeloid leukemia (AML) cells. The effect of Salinosporamide A in combination with MS-275 was compared to the effect of Velcade in combination with MS-275. The combination of Salinosporamide A with MS-275 had a greater synergistic effect compared to bortezomib (Velcade) with MS-275 (Figure 8). These findings demonstrate that combination of HDACi, (MS-275), with low doses of Salinosporamide A results in synergistic induction of apoptosis and these effects are more potent than those seen when bortezomib is combined with the same agents (Figure 8).

[0467] Salinosporamide A enhances the activity of vorinostat (SAHA) in Hodgkin's Lymphoma cell lines. Human Reed-Sternberg cell lymphoma (HD-LM2) were incubated with SAHA (1 μ M), Salinosporamide A (10 nM) and a combination of SAHA (1 μ M) and Salinosporamide A (10 nM). HD-LM2 cell viability was analyzed at 24 hours and 48 hours (Figure 9). The combination of SAHA and Salinosporamide A showed statistical significance at both 24 hours and 48 hours. Similar synergistic results were obtained with Hodgkin's L428 (figure 10A) and KM-H2 (Figure 10B) cell lines.

[0468] Antitumor activity of Salinosporamide A and MS-275 in a human myeloma cell line (RPMI 8226 MM cells) was examined (Figure 11). The effect of combinations of Salinosporamide A (14 nM) with MS-275 (0.5 μ M, 2 μ M and 3 μ M) were found to induce synergism. Additionally, the effect of combination of Salinosporamide A (18 nM) with MS-275 (1 μ M) and Salinosporamide A (16 nM) with MS-275 (1.5 μ M) were also found to induce synergism. Results are shown in Table 1 below.

Table 1

MS-275 Dose (μ M)	Salinosporamide A Dose (nM)	Mixture Effect	CI
0.5	14	0.26	0.20
1	18	0.30	0.31
1.5	16	0.34	0.38
2	14	0.36	0.45
3	14	0.40	0.54

[0469] Antitumor activity of Salinosporamide A and MS-275 in a human myeloma cell line (OPM-1 MM cells) was examined (Figure 12). The effect of combinations of Salinosporamide A (12 nM, 14 nM and 18 nM) with MS-275 (1.5 μ M) were found to induce synergism. Additionally, the effect of combination of Salinosporamide A (18 nM and 20 nM) with MS-275 (2 μ M) was also found to induce synergism. The lowest dosage combination, Salinosporamide A (12 nM) and MS-275 (1.5 μ M), greatest synergistic effect (CI=0.65). The highest dosage combination, Salinosporamide A (20 nM) and MS-275 (2

μM), had the lowest synergistic effect ($\text{CI}=0.96$). This highest dosage combination had a nearly additive effect. Results are show Table 2 below.

Table 2

MS-275 Dose (μM)	Salinosporamide A Dose (nM)	Mixture Effect	CI
1.5	12	0.53	0.65
1.5	14	0.53	0.73
1.5	18	0.54	0.82
2	18	0.54	0.89
2	20	0.54	0.96

[0470] Antitumor activity of Salinosporamide A and MS-275 in a human myeloma cell line (DHL-6 MM cells) was examined (Figure 13). The effect of combinations of Salinosporamide A (16 nM and 18 nM) with MS-275 (0.5 μM , 1 μM , 1.5 μM and 2 μM) were found to induce synergism. The lowest dosage combination ($\text{CI}=0.89$), Salinosporamide A (16 nM) and MS-275 (0.5 μM), had nearly the same synergistic effect as the highest dosage combination ($\text{CI}=0.92$), Salinosporamide A (18 nM) and MS-275 (2 μM). Results are show Table 3 below.

Table 3

MSX- 275 Dose (μM)	Salinosporamide A Dose (nM)	Mixture Effect	CI
0.5	16	0.62	0.89
1	16	0.66	0.88
	16	0.64	0.93
1.5	18	0.81	0.90
2	18	0.75	0.92

[0471] Antitumor activity of Salinosporamide A and MS-275 in a human myeloma cell line (Dox-6 MM cells) was examined (Figure 14). The effect of combinations of Salinosporamide A (12 nM, 14 nM, and 20 nM) with MS-275 (0.5 μ M, 1 μ M, 1.5 μ M and 2 μ M) were found to induce synergism. The lowest dosage of MS-275 (0.5 μ M) with the highest dosage of Salinosporamide A (20 nM) had the greatest synergistic effect (CI=0.30). The highest dosage of MS-275 (2 μ M) with the lower dosages of Salinosporamide A (12 nM and 14 nM) had lower synergistic effects (CI=0.73 and 0.79, respectively). Results are shown in Table 4 below.

Table 4

MS- 275 Dose (μ M)	Salinosporamide A Dose (nM)	Mixture Effect	CI
0.5	20	0.64	0.30
1	20	0.80	0.51
1.5	14	0.92	0.65
2	12	0.97	0.73
2	14	0.95	0.79

[0472] Antitumor activity of Salinosporamide A and MS-275 in a human myeloma cell line (Dox-40 MM cells) was examined (Figure 15). The effect of combinations of Salinosporamide A (14 nM, 16 nM, and 20 nM) with MS-275 (0.5 μ M, 1 μ M, 1.5 μ M and 2 μ M) were observed. It was found that Salinosporamide A (14 nM) with MS-275 (0.5 μ M) had the largest synergistic effect. At the higher dosage combinations of Salinosporamide A and MS-275 the effects were nearly additive (CI close to 1). Results are shown in Table 5 below.

Table 5

MS-275 Dose (μ M)	Salinosporamide A Dose (nM)	Mixture Effect	CI
0.5	14	0.70	0.76
1	20	0.77	0.98
1.5	16	0.78	0.98
1.5	20	0.81	0.98
2	16	0.81	0.98
2	20	0.84	0.96

[0473] Antitumor activity of Salinosporamide A and MS-275 in a human myeloma cell line (LR-5 MM cells) was examined (Figure 16). The effect of combinations of Salinosporamide A (12 nM, 14 nM, 18 nM and 20 nM) with MS-275 (0.5 μ M, 1 μ M, and 2 μ M) were observed. It was found that Salinosporamide A (12 nM and 14 nM) with MS-275 (0.5 μ M, 1 μ M and 2 μ M) had the largest synergistic effect. The higher dosages of Salinosporamide A (18 nM and 20 nM) with MS-275 (1 μ M) had lower synergistic effects. The synergistic effect at the lower dosages of Salinosporamide A (12 nM and 14 nM) was nearly double that of the higher dosages of Salinosporamide A (18 nM and 20 nM). Results are shown in Table 6 below.

Table 6

MS-275 Dose (μ M)	Salinosporamide A Dose (nM)	Mixture Effect	CI
0.5	12	0.44	0.23
1	14	0.45	0.27
1	18	0.41	0.72
1	20	0.42	0.63
2	12	0.46	0.31

[0474] Antitumor activity of Salinosporamide A and MS-275 in a human myeloma cell line (MM.1R cells) was examined (Figure 17). The effect of combinations of Salinosporamide A (3 nM, 5 nM, and 7 nM) with MS-275 (0.25 μ M, 0.5 μ M, and 1 μ M) were observed. The effect of all combinations of Salinosporamide A with MS-275 were found to induce synergism. Although, the combination of Salinosporamide A (3 nM) with MS-275 (1 μ M) was found to be nearly additive (CI=0.91). Results are shown in Table 7 below.

Table 7

MS-275 Dose (μ M)	Salinosporamide A Dose (nM)	Mixture Effect	CI
0.25	5	0.66	0.72
0.25	7	0.72	0.64
0.50	5	0.77	0.70
1	3	0.82	0.91
1	7	0.85	0.83

EXAMPLE 38

EFFECT OF SALINOSPORAMIDE A AND COMBINATIONS WITH HDAC
INHIBITORS ON SOLID TUMORS

[0475] Activity of Salinosporamide A and vorinostat on SB-2 (Figure 18A) and WM-266-4 (Figure 18B) melanoma cell lines was observed. The combination of Salinosporamide A (10 nM) with vorinostat (0.5 μ M) was compared to Salinosporamide A (10 nM), vorinostat (0.5 μ M) and control in the SB-2 and WM-266-4 melanoma cell lines. In the SB-2 melanoma cell line Salinosporamide A (10 nM) and vorinostat (0.5 μ M) had nearly the same activity as the control. However, when Salinosporamide A (10 nM) and vorinostat (0.5 μ M) were used in combination enhanced activity was observed. In the WM-266-4 melanoma cell line Salinosporamide A (10 nM) and vorinostat (0.5 μ M) showed activity greater than the control. Additionally, when Salinosporamide A (10 nM) and vorinostat (0.5 μ M) were used in combination synergistic activity was observed. Activity of

Salinosporamide A and vorinostat on MeWo melanoma cell lines was observed (Figure 19). The combination of Salinosporamide A with vorinostat was compared to Salinosporamide A, vorinostat and control in the MeWo melanoma cell lines, the combination of Salinosporamide A (10 nM) with vorinostat (5 μ M) increases apoptosis in the MeWo melanoma cell line compared to treatment with the individual agents alone (Figure 19).

[0476] The sensitivity of human lung carcinoma cell lines to Salinosporamide A is shown in Table 8. The IC₅₀ values show that Salinosporamide A is active against a variety of human lung tumor types and cell lines. Salinosporamide A was tested at 5 nM to 1 μ M and cell growth assessed by MTS assays.

Table 8

Human Lung Tumor Type	Cell Line	IC50 \pm SD (nM)
Large Cell Carcinoma	H1341	10 \pm 1
Small Cell Carcinoma	H196	20 \pm 1
Large Cell Carcinoma	H157	30 \pm 2
Squamous Cell Carcinoma	H226	30 \pm 3
Adenocarcinoma	H441	50 \pm 6
Adenocarcinoma	A549	160 \pm 20
Bronchioloalveolar Carcinoma	H322C	280 \pm 22
Adenocarcinoma	HCC4006	300 \pm 30

[0477] The IC₅₀ for Salinosporamide A used alone ranges from about 10 nM (large cell carcinoma H1341 cell line) to about 300 nM (adenocarcinoma HCC4006 cell line). The lung carcinoma cell lines were treated with Salinosporamide A at doses of 5 nM to 500 nM as single agent or in combination with vorinostat (SAHA) (2 μ M). At the doses tested, the results indicate that the combination of vorinostat (SAHA) plus Salinosporamide A has additive effects on growth inhibition in the lung cancer cell lines tested (Figures 20 and 21). Additionally, isobologram analysis show the effect of combinations of vorinostat (SAHA) plus Salinosporamide A in various lung carcinoma cell lines: H441 (Figure 22A); A549

(Figure 22B); H322C (Figure 22C); HCC4006 (Figure 22D); H1341 (Figure 22E); H196 (Figure 22F); H157 (Figure 22G); and H226 (Figure 22 H).

[0478] The resistance of human pancreatic carcinoma cell lines to gemcitabine is disclosed (Figure 23). The results indicate that human pancreatic carcinoma cell lines are resistant to treatment with gemcitabine alone. However, when gemcitabine (1 μ M or 10 μ M) is used at in combination with Salinosporamide A a dosage effect is observed.

[0479] The combination of Salinosporamide A with vorinostat increases apoptosis in human pancreatic carcinoma cell lines (MPanc 96) compared to treatment with the individual agents alone (Figure 24). The results indicate that the combination of Salinosporamide A (20 μ M) and vorinostat (10 μ M) increased apoptosis more than the combination of Salinosporamide A (50 μ M) and vorinostat (10 μ M). Increasing the amount of Salinosporamide A with vorinostat kept at a constant dosage (10 μ M) did not continue the trend of increasing apoptosis.

EXAMPLE 39

MECHANISMS OF ACTION OF SALINOSPORAMIDE A, HDAC INHIBITORS AND COMBINATIONS OF OF SALINOSPORAMIDE A WITH HDAC INHIBITORS

[0480] The HDAC inhibitor MS-275 can decrease mRNA expression of 20S proteasome β subunits in Jurkat cells (Figure 25). The relative expression of 20S proteasome β 1, β 2, and β 5 subunits were all decreased when treated with MS-275 (5 μ M) for 24 hours. The HDAC inhibitor vorinostat can decrease mRNA expression of 20S proteasomal β 5 subunit in Jurkat cells as measured at 12 hours and 18 hours (Figure 26). The expression of β 5 mRNA was analyzed by real time PCR were all decreased when treated with MS-275 (5 μ M) for 24 hours.

[0481] The HDAC inhibitor MS-275 in combination with Salinosporamide A or bortezomib causes Histone-3 to hyperacetylate (Figure 27). Jurkat T-cells were treated for 6 hours with MS-275 (5 μ M), Salinosporamide A (10 nM), bortezomib (10 nM) and combinations of MS-275 (5 μ M) with Salinosporamide A (10 nM) or bortezomib (10 nM). It can be seen from the data that the combination treatments cause an increase in the acetylation

of Ac-H3 and H3. Additionally, the data shows that Salinosporamide A (10 nM) causes an increase in the acetylation of H3 relative to control.

[0482] The combination of Salinosporamide A with vorinostat causes hyperacetylation of Histone-3 in jurkat cells (Figure 28). The data shows that N-acetylcysteine (NAC) inhibits the hyperacetylation of Histone-3 in jurkat cells.

[0483] Histone-3 ubiquitination is not affected by NPI-0052 or the combination with MS-275 (Figure 29).

[0484] Synergistic and additive effects of vorinostat pretreatment of human Jurkat ALL cells followed by treatment with bortezomib has been studied (Figure 30). Additionally, synergistic and additive effects of simultaneous treatment with vorinostat and bortezomib has been studied (Figure 31). Synergistic and additive effects of vorinostat pretreatment followed by Salinosporamide A has been studied (Figure 32). Additionally, synergistic and additive effects of simultaneous treatment with vorinostat and Salinosporamide A has been studied (Figure 33). The data shows that treatment of human Jurkat ALL cells with vorinostat and Salinosporamide A produces a greater synergistic effect when the cells are pretreated with vorinostat than when simultaneously treated with vorinostat and Salinosporamide A.

[0485] The effect of MS-275 and Salinosporamide A on superoxide levels in Jurkat T-cells has been studied (Figure 34). Relative to control MS-275 (5 μ M) does not increase superoxide levels based on mean fluorescence. Salinosporamide A (10 nM) does increase superoxide levels based on mean fluorescence. The combination of MS-275 (5 μ M) and Salinosporamide A (10 nM) provides a synergistic increase in superoxide levels based on mean fluorescence.

[0486] The effect of MS-275 and Salinosporamide A on superoxide levels in Caspase-8 deficient cells has been studied (Figure 35). Relative to control MS-275 (5 μ M) does not increase superoxide levels substantially based on mean fluorescence. Salinosporamide A (10 nM) does increase superoxide levels based on mean fluorescence. The combination of MS-275 (5 μ M) and Salinosporamide A (10 nM) provides a synergistic increase in superoxide levels based on mean fluorescence.

[0487] The HDAC inhibitor vorinostat in combination with Salinosporamide A or bortezomib causes formation of reactive oxygen species (ROS) (Figure 36). When N-

acetylcysteine (NAC) is included in the combinations the amount of ROS decrease as measured by mean fluorescence. N-acetylcysteine (NAC) decreases formation of ROS when combined with vorinostat and Salinosporamide A but not vorinostat and bortezomib (Figure 37).

[0488] Caspase-8 Activation is required for synergistic apoptosis by Salinosporamide A and vorinostat in ALL Cells (Figure 38). The results show that the combination of vorinostat and Salinosporamide A does not induce apoptosis in cells that are caspase-8 deficient as strongly as cells that are not caspase-8 deficient.

[0489] Regulation of NF- κ B Activity *in vitro* by vorinostat and Salinosporamide A combinations (Figure 39). Regulation of NF- κ B in human pancreatic carcinoma cells by Salinosporamide A and vorinostat (Figure 40).

EXAMPLE 40

ACTIVITY OF SALINOSPORAMIDE A AND VORINOSTAT COMBINATIONS IN A DRUG RESISTANT ORTHOTOPIC PANCREATIC CARCINOMA TUMOR MODEL

[0490] The combination of Salinosporamide A and vorinostat exhibit enhanced activity in an orthotopic pancreatic tumor model (Figure 41). mPANC-96 is a drug-resistant mesenchymal pancreatic tumor. Luciferase-transduced mPANC-96 cells were used to generate orthotopic tumors in nude mice. The tumor volumes were monitored by bioluminescence imaging after injection of luciferin. Salinosporamide A was administered IP at 0.15 mg/kg weekly. Vorinostat was administered IP 5 days/week at 50 mg/kg. Tumor measurements were made after 3 weeks of therapy. The combination of Salinosporamide A (0.15 mg/kg weekly) and Vorinostat (5 days/week at 50 mg/kg) showed enhanced reduction in tumor volume compared to control.

[0491] The examples described above are set forth solely to assist in the understanding of the embodiments. Thus, those skilled in the art will appreciate that the methods may provide derivatives of compounds.

[0492] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well

as those inherent therein. The methods and procedures described herein are presently representative of preferred embodiments and are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention.

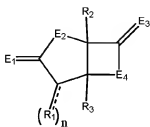
[0493] It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the embodiments disclosed herein without departing from the scope and spirit of the invention.

[0494] All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

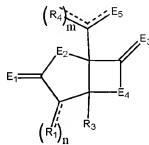
[0495] The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions indicates the exclusion of equivalents of the features shown and described or portions thereof. It is recognized that various modifications are possible within the scope of the invention. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed can be resorted to by those skilled in the art, and that such modifications and variations are considered to be falling within the scope of the embodiments of the invention.

WHAT IS CLAIMED IS:

1. A method of treating cancer comprising administering to an animal a compound having the structure of any one of Formulas I and II, or a pharmaceutically acceptable salt or pro-drug thereof:



Formula I



Formula II

in combination with a histone deacetylase inhibitor (HDACi);

wherein.

the dashed lines represent a single or a double bond;

each **R₁** is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

n is 1 or 2, where if **n** is 2, then each **R₁** can be the same or different;

m is 1 or 2, where if **m** is 2, then each **R₄** can be the same or different,

R₂ is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide,

sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

R₃ is a halogen or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

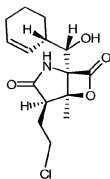
each of **E₁**, **E₃**, **E₄** and **E₅** is an optionally substituted heteroatom;

E₂ is an optionally substituted heteroatom or -CH₂- group; and

each **R₄** is separately a halogen, a cyano, a nitro, an azido, or a thiocyno, or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

2. The method of Claim 1, wherein the cancer is selected from the group consisting of breast cancer, sarcoma, leukemia, uretal cancer, bladder cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, brain cancer and central nervous system (CNS) cancer.

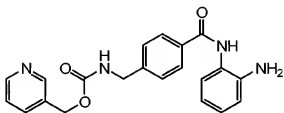
3. The method of any one of the preceding claims, wherein the compound is Salinosporamide A;



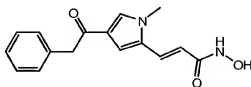
Salinosporamide A

4. The method of any one of the preceding claims, wherein the HDACi and the compound having the structure of any one of Formulas I and II work in a synergistic manner to treat cancer.

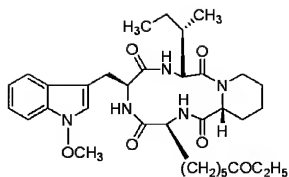
5. The method of any one of the preceding claims, wherein the HDACi is selected from the group consisting of:



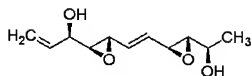
(pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate,



APHA compound 8,



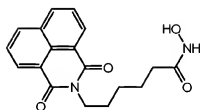
apicidin,



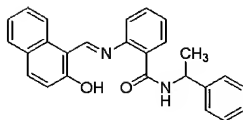
(-)-Depudecin,



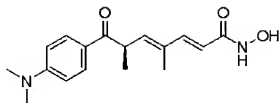
sodium Butyrate,



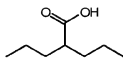
Scriptaid,



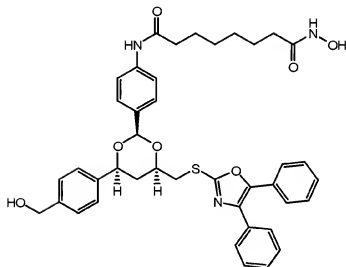
Sirtinol,



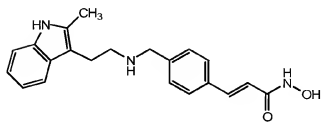
trichostatin A,



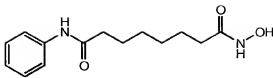
valproic acid,



tubacin,

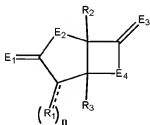


panobinostat, and



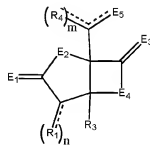
vorinostat (suberoylanilide hydroxamic acid (SAHA)).

6. The method of Claim 5, wherein the HDACi is (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275).
7. The method of Claim 5, wherein the HDACi is valproic acid.
8. The method of Claim 5, wherein the HDACi is vorinostat.
9. The method of any one of the preceding claims, wherein the cancer is leukemia.
10. The method of any one of claims 1-8, wherein the cancer is lymphoma.
11. The method of any one of the preceding claims, wherein the cancer comprises a tumor.
12. The method of Claim 11, wherein the tumor is a refractory solid tumor.
13. The method of any one of the preceding claims, further comprising co-administering a chemotherapeutic agent.
14. The method of any one of the preceding claims, wherein the animal is a human.
15. A pharmaceutical composition comprising a histone deacetylase inhibitor (HDACi) and a compound of any one of Formulas I and II:



Formula I

and



Formula II

wherein:

the dashed lines represent a single or a double bond;

each **R₁** is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyanato, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

n is 1 or 2, where if **n** is 2, then each **R₁** can be the same or different;

m is 1 or 2, where if **m** is 2, then each **R₄** can be the same or different;

R₂ is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyanato, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

R₃ is a halogen or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

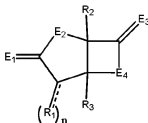
each of **E₁**, **E₃**, **E₄** and **E₅** is an optionally substituted heteroatom;

E₂ is an optionally substituted heteroatom or -CH₂- group; and

each **R₄** is separately a halogen, a cyano, a nitro, an azido, or a thiocyanato, or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl,

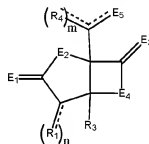
alkoxycarbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

16. The composition of Claim 15, wherein the compound is Salinosporamide A.
17. The composition of Claim 15 or 16, wherein the HDACi is selected from the group consisting of (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275), APHA compound 8, Apicidin, (-)-Depudecin, sodium butyrate, scriptaid, sirtinol, trichostatin A, valproic acid and vorinostat.
18. The composition of Claim 17, wherein the HDACi is (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275).
19. The composition of Claim 17, wherein the HDACi is valproic acid.
20. The composition of Claim 17, wherein the HDACi is vorinostat.
21. A method of inhibiting the growth of a cancer cell comprising contacting the cell with a HDACi and a compound having the structure of any one of Formulas I and II:



Formula I

and



Formula II

wherein:

the dashed lines represent a single or a double bond;

each R_1 is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocarbonyl, or selected from the group consisting of optionally substituted: C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio,

oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

n is 1 or 2, where if **n** is 2, then each **R₁** can be the same or different;

m is 1 or 2, where if **m** is 2, then each **R₁** can be the same or different;

R₂ is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyno, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

R₃ is a halogen or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

each of **E₁**, **E₃**, **E₄** and **E₅** is an optionally substituted heteroatom;

E₂ is an optionally substituted heteroatom or -CH₂- group; and

each **R₄** is separately a halogen, a cyano, a nitro, an azido, or a thiocyno, or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

22. The method of Claim 21, wherein the compound is Salinosporamide A.

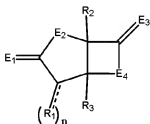
23. The method of Claim 21 or 22, wherein the HDACi is selected the group consisting of (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275), APHA compound 8, apicidin, (-)-Depudecin, sodium butyrate, scriptaid, sirtinol, trichostatin A, valproic acid and vorinostat.

24. The method of Claim 23, wherein the HDACi is (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275).

25. The method of Claim 23, wherein the HDACi is valproic acid.

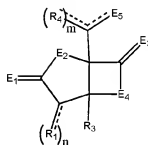
26. The method of Claim 23, wherein the HDACi is vorinostat.

27. A method of inducing apoptosis of a cancer cell comprising contacting the cell with a HDACi and a compound having the structure of any one of Formulas I and II:



Formula I

and



Formula II

wherein:

the dashed lines represent a single or a double bond;

each **R₁** is separately a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyanato, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

n is 1 or 2, where if **n** is 2, then each **R₁** can be the same or different;

m is 1 or 2, where if **m** is 2, then each **R₄** can be the same or different;

R₂ is a hydrogen, a halogen, a cyano, a nitro, an azido, a hydroxy, or a thiocyanato, or selected from the group consisting of optionally substituted: C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl;

R₃ is a halogen or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, aminocarbonyl, aminocarbonyloxy, nitro, azido, phenyl, cycloalkylacyl, hydroxy, alkylthio, arylthio, oxysulfonyl, carboxy, cyano, and halogenated alkyl including polyhalogenated alkyl;

each of **E₁**, **E₃**, **E₄** and **E₅** is an optionally substituted heteroatom;

E₂ is an optionally substituted heteroatom or -CH₂- group; and

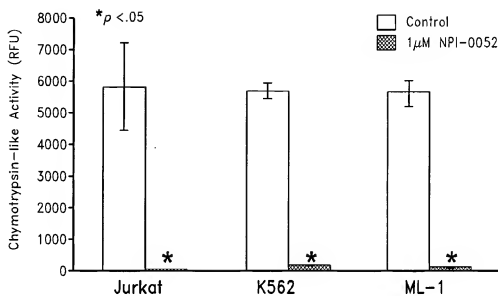
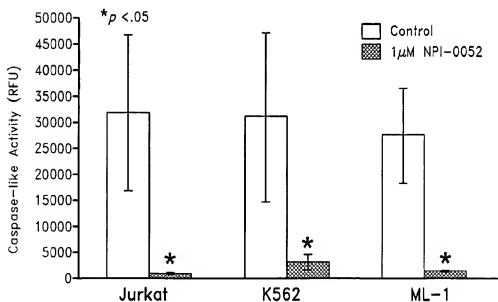
each **R₄** is separately a halogen, a cyano, a nitro, an azido, or a thiocyanato, or selected from the group consisting of optionally substituted C₁-C₂₄ alkyl, C₂-C₂₄ alkenyl, C₂-C₂₄ alkynyl, acyl, acyloxy, alkyloxycarbonyloxy, aryloxycarbonyloxy, cycloalkyl, cycloalkenyl, alkoxy, cycloalkoxy, aryl, heteroaryl, arylalkoxycarbonyl, alkoxy carbonylacyl, amino, hydroxy, aminocarbonyl, aminocarbonyloxy, phenyl, cycloalkylacyl, alkylthio, arylthio, oxysulfonyl, carboxy, thio, sulfoxide, sulfone, sulfonate esters, boronic acids and esters, and halogenated alkyl including polyhalogenated alkyl.

28. The method of Claim 27, wherein the compound is Salinosporamide A.

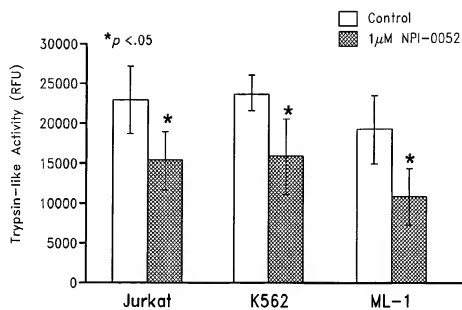
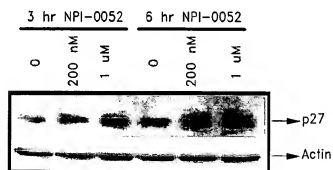
29. The method of Claim 27 or 28, wherein the HDACi is selected the group consisting of (pyridin-3-yl)methyl 4-(2-aminophenylcarbomoyl)benzylcarbamate (MS-275), APHA compound 8, Apicidin, (-)-Depudecin, sodium butyrate, scriptaid, sirtinol, trichostatin A, valproic acid and vorinostat.

30. The method of Claim 29, wherein the HDACi is (pyridin-3-yl)methyl 4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275).
31. The method of Claim 29, wherein the HDACi is valproic acid.
32. The method of Claim 29, wherein the HDACi is vorinostat.

1/61

*FIG. 1A**FIG. 1B*

2/61

*FIG. 1C**FIG. 1D*

3/61

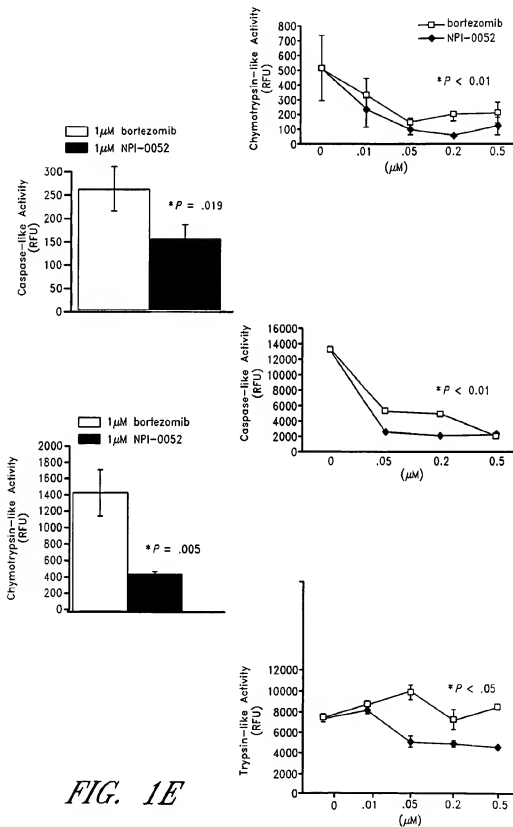


FIG. 1E

4/61

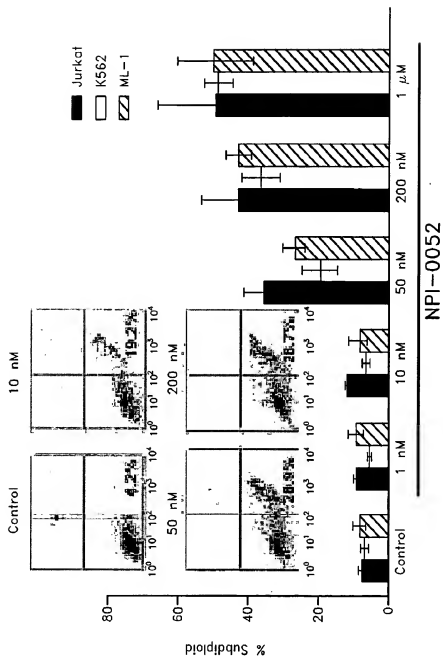


FIG. 2A

5/61

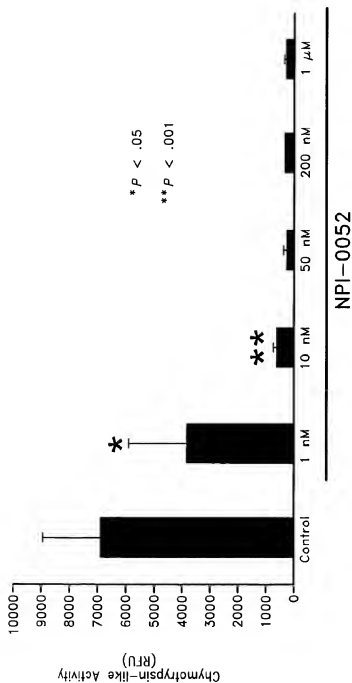


FIG. 2B

6/61

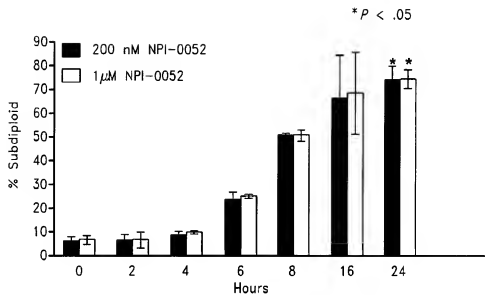


FIG. 2C

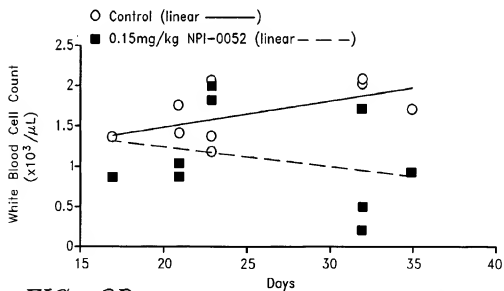


FIG. 2D

7/61

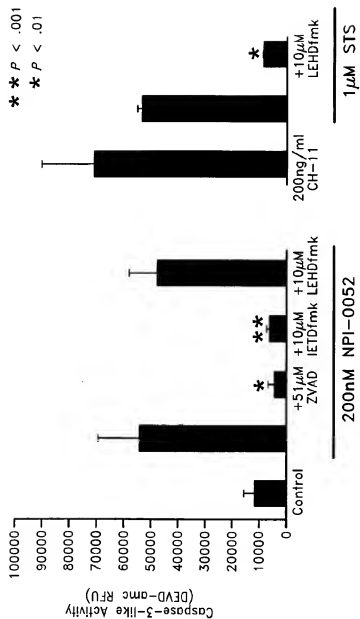


FIG. 3A

8/61

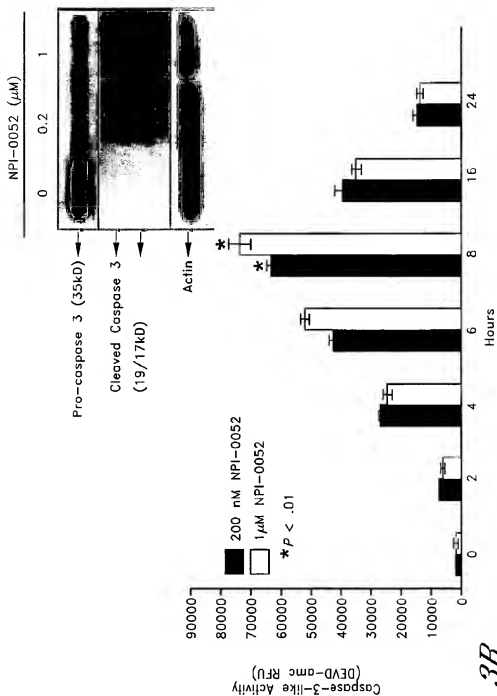


FIG. 3B

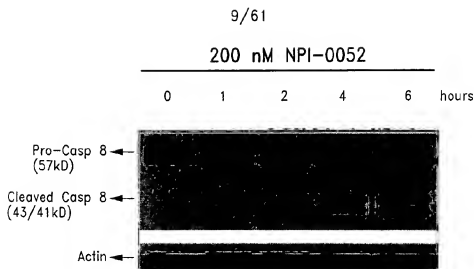


FIG. 3C

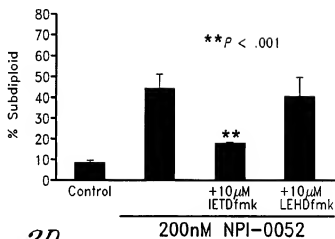


FIG. 3D

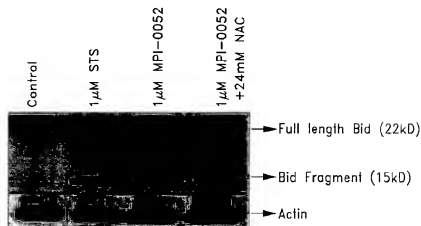
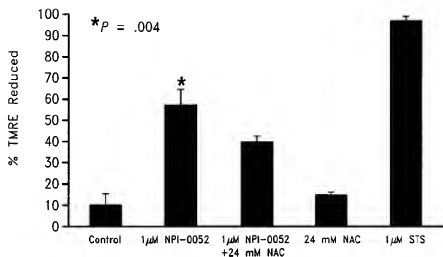
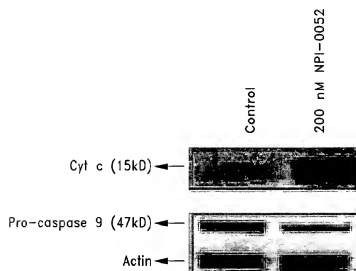


FIG. 3D

10/61

*FIG. 3F**FIG. 3G*

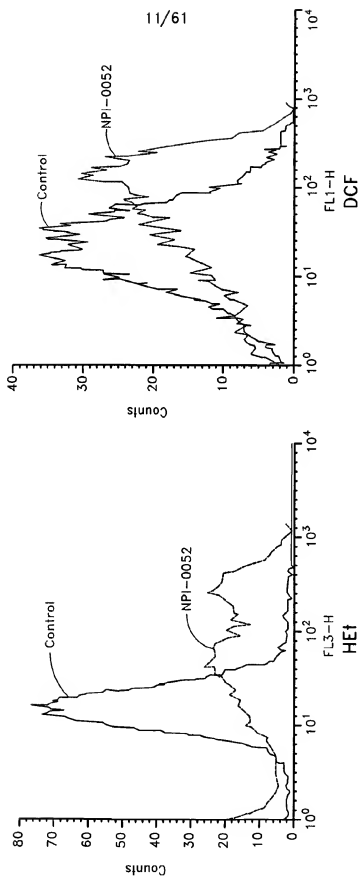


FIG. 3H

12/61

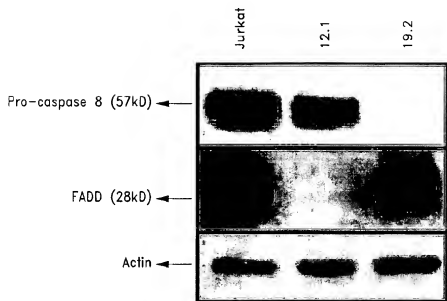


FIG. 4A

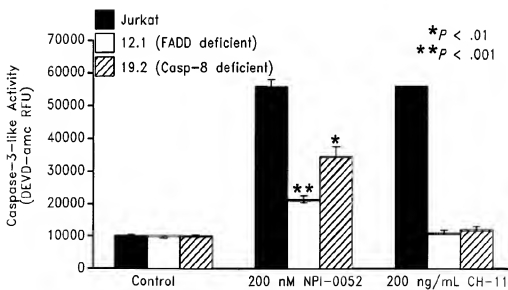


FIG. 4B

13/61

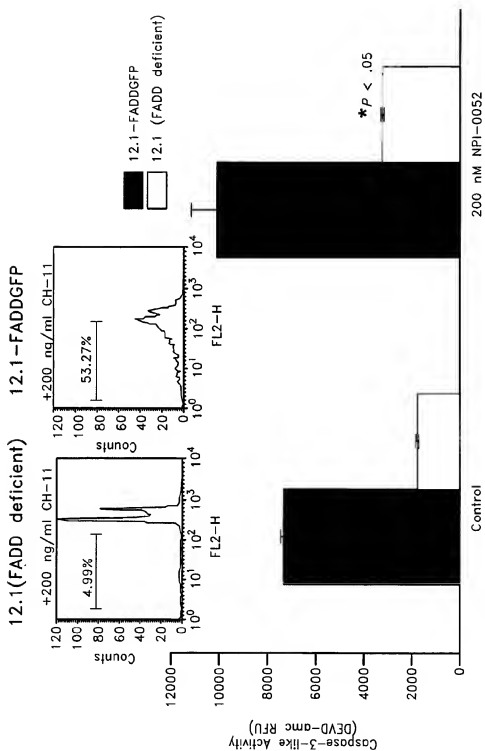


FIG. 4C

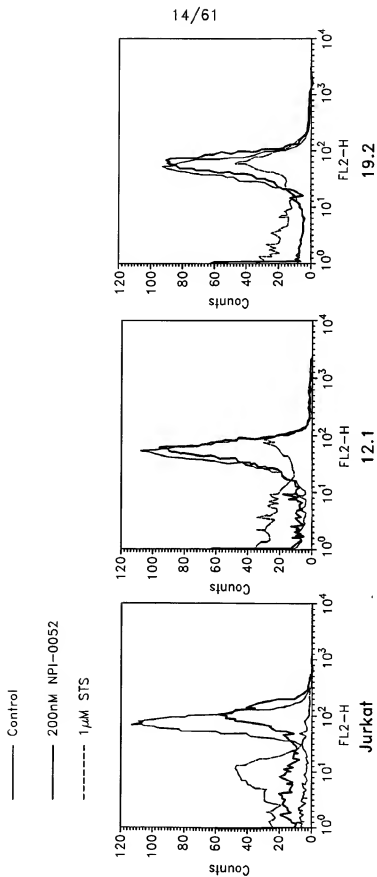


FIG. 4D

15/61

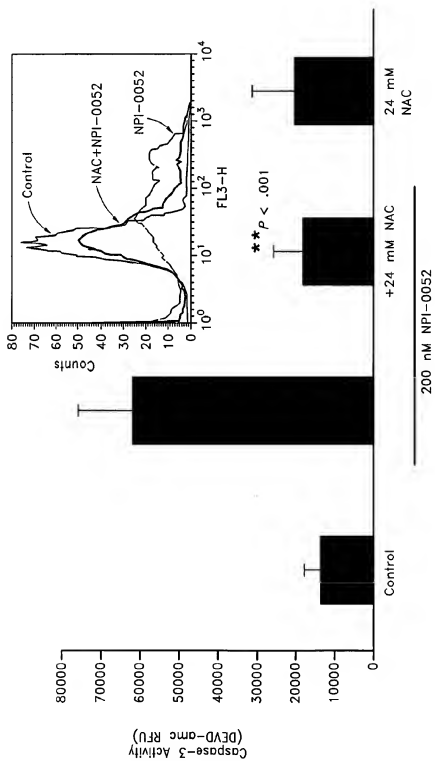
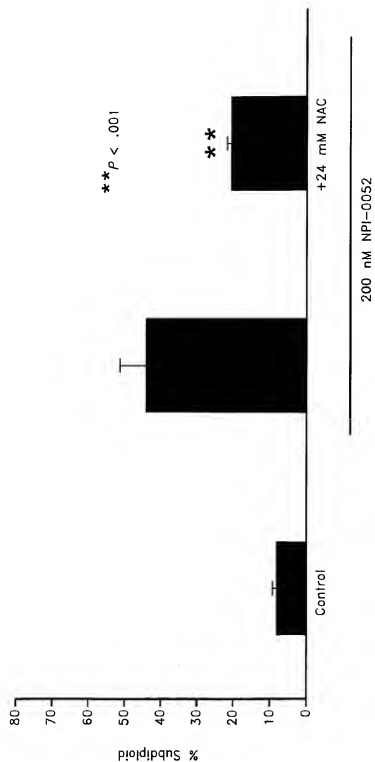
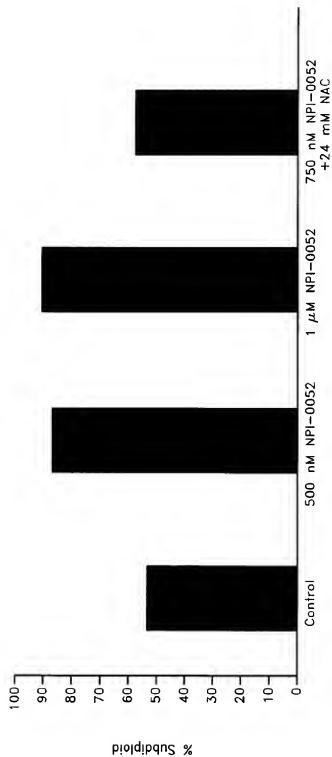


FIG. 5A

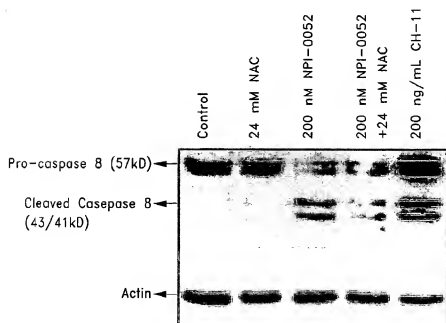
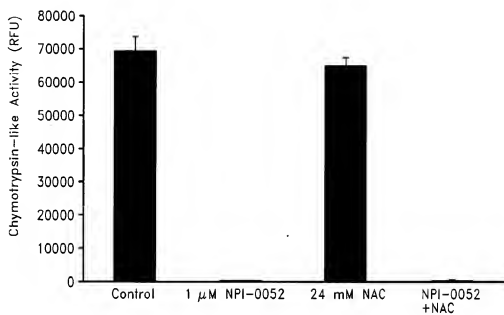
16/61

*FIG. 5B*

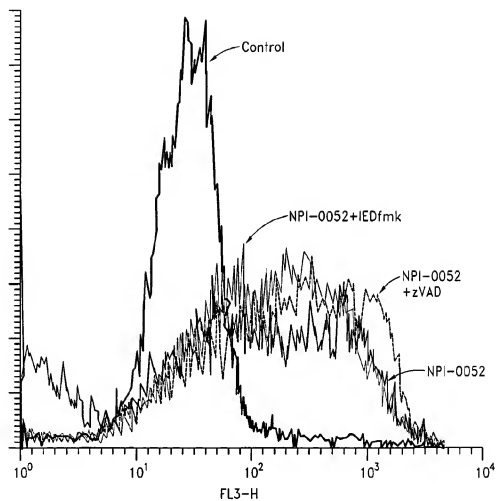
17/61

*FIG. 5C*

18/61

*FIG. 5E*

19/61

*FIG. 5F*

20/61

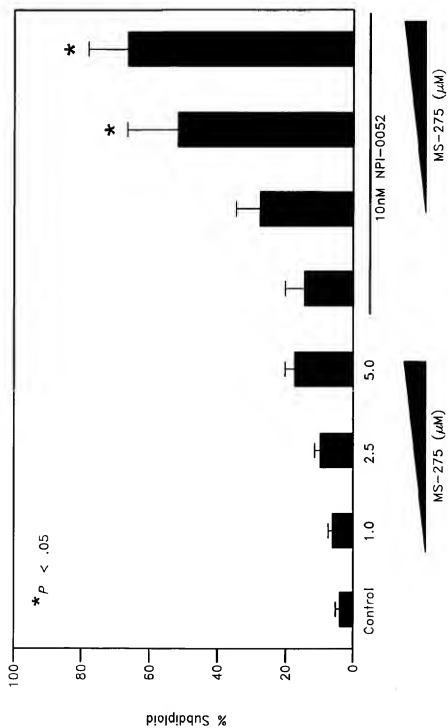


FIG. 6A

21/61

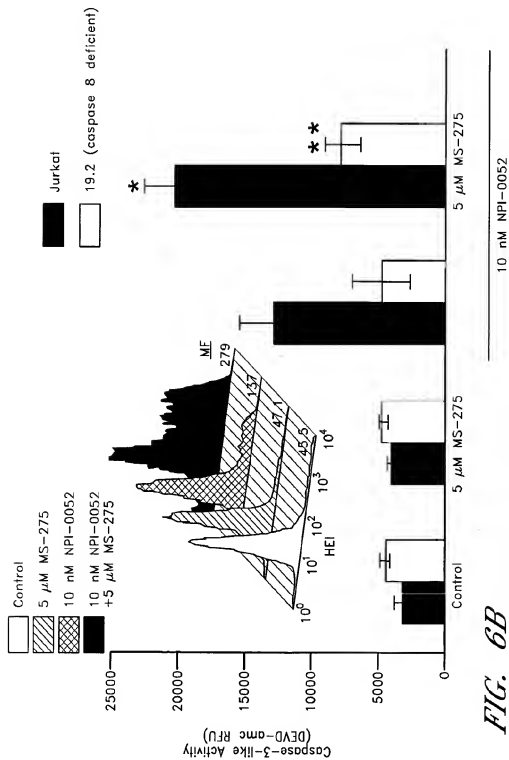
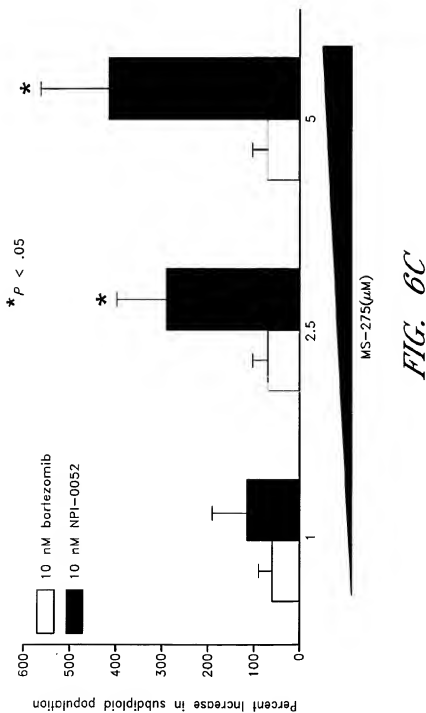


FIG. 6B

22/61



23/61

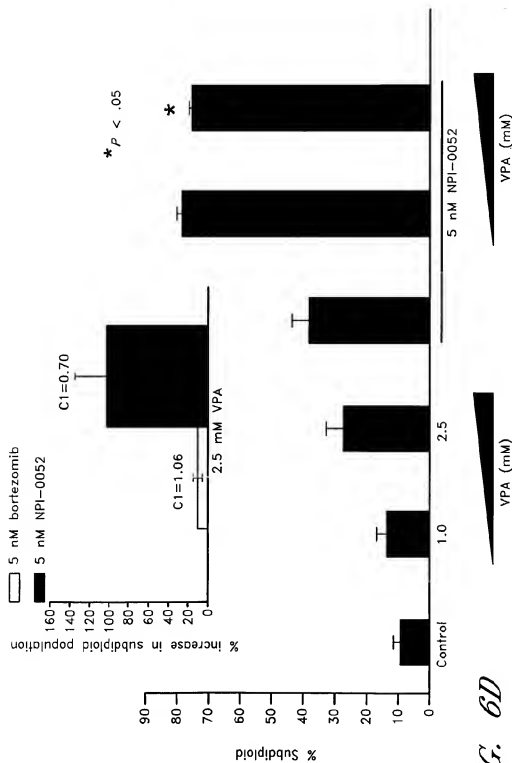
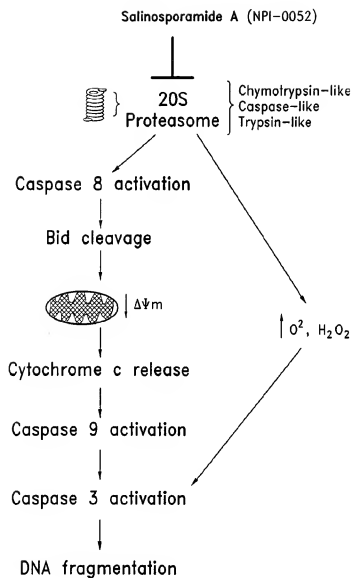


FIG. 6D

24/61

*FIG. 7*

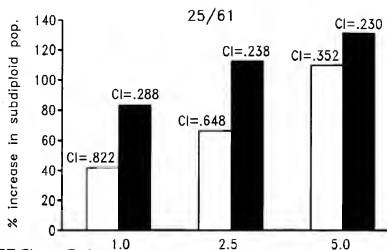


FIG. 8A

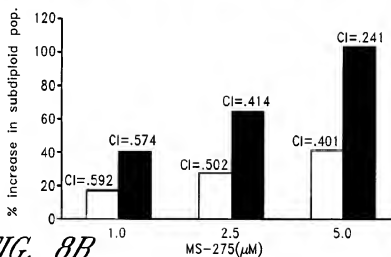


FIG. 8B

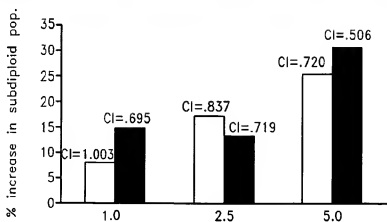


FIG. 8C

□ bortezomib

■ Salinosporamide A

A-1nM B-5nM C-10nM

Synergy combination index (CI) < 1

Additive effect CI = 1

Antagonism CI > 1

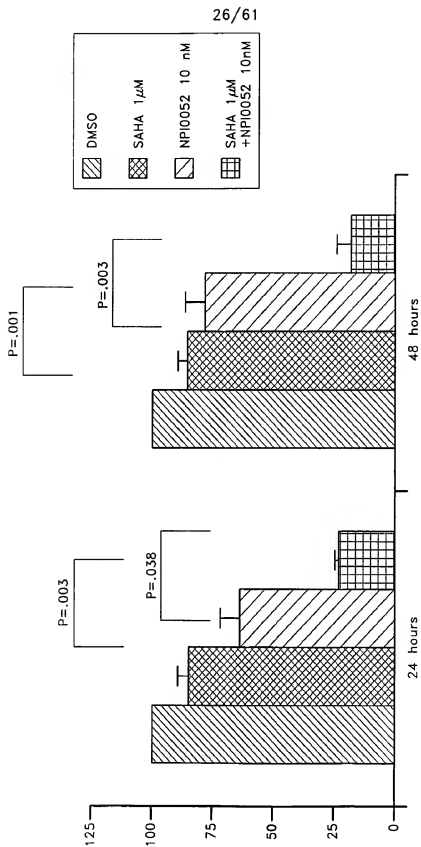
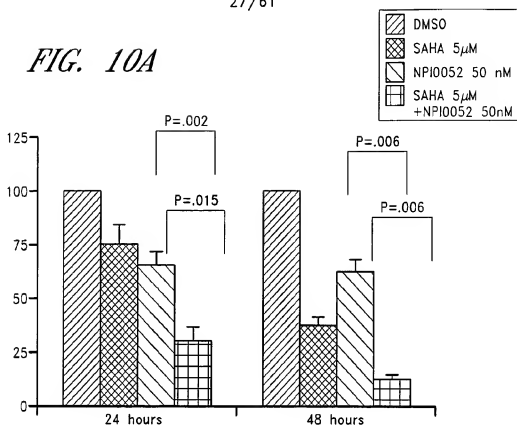
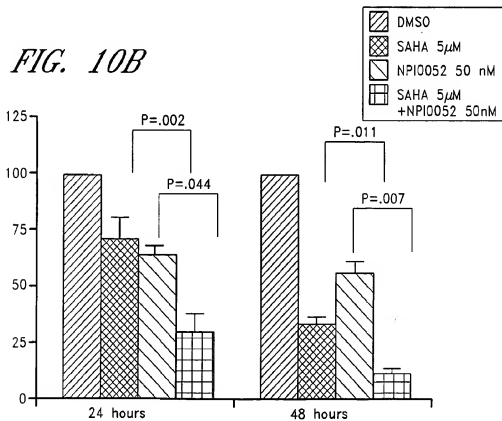


FIG. 9

27/61

FIG. 10A*FIG. 10B*

28/61

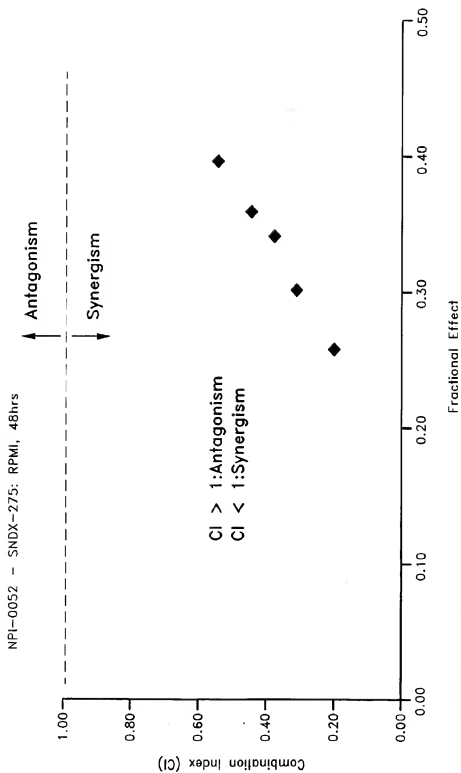


FIG. 11

29/61

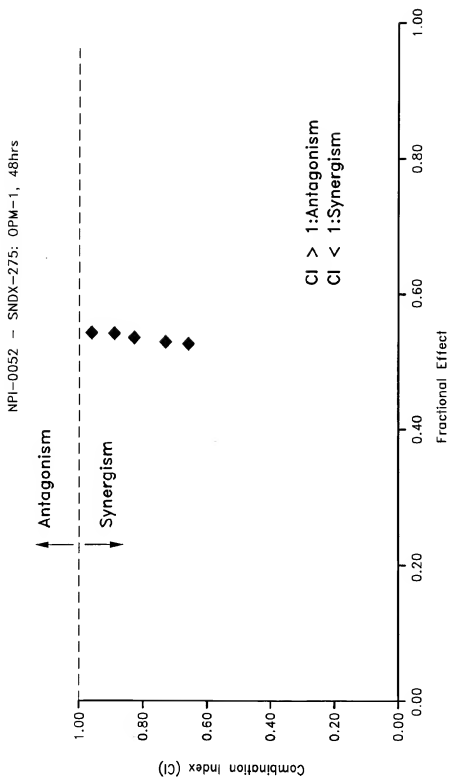


FIG. 12

30/61

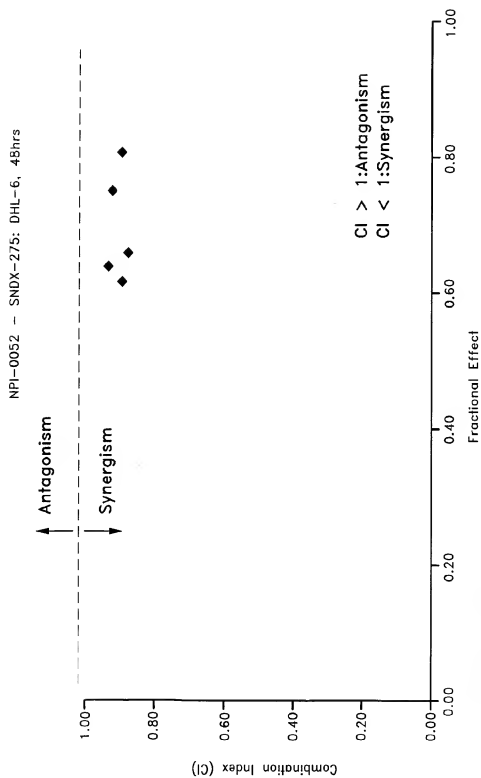


FIG. 13

31/61

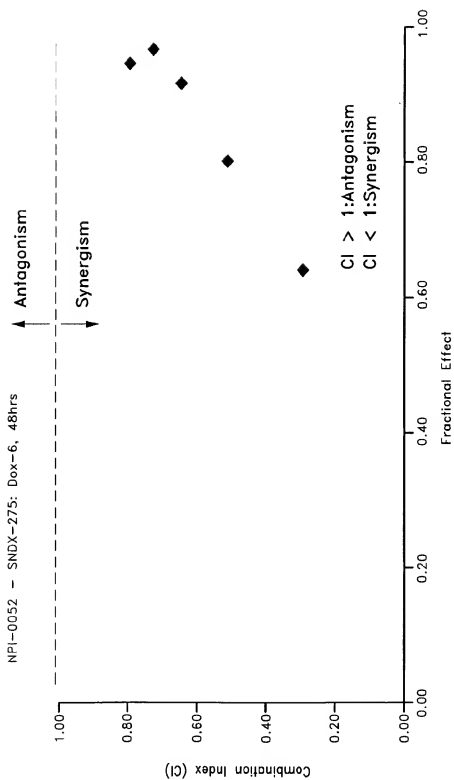


FIG. 14

32/61

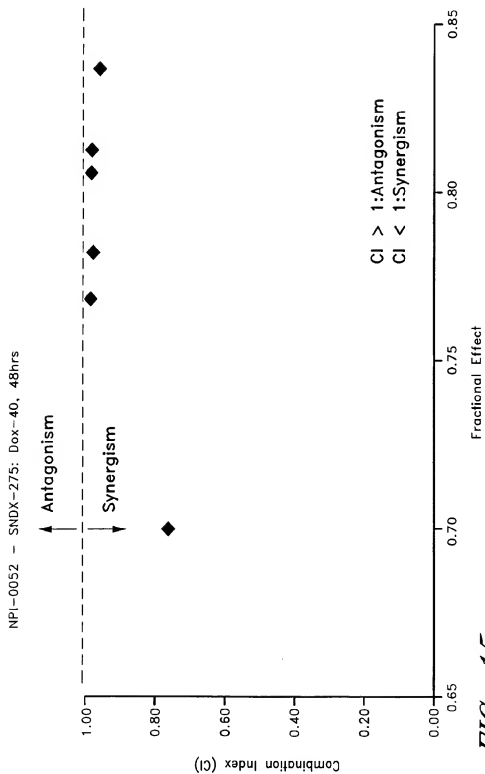


FIG. 15

33/61

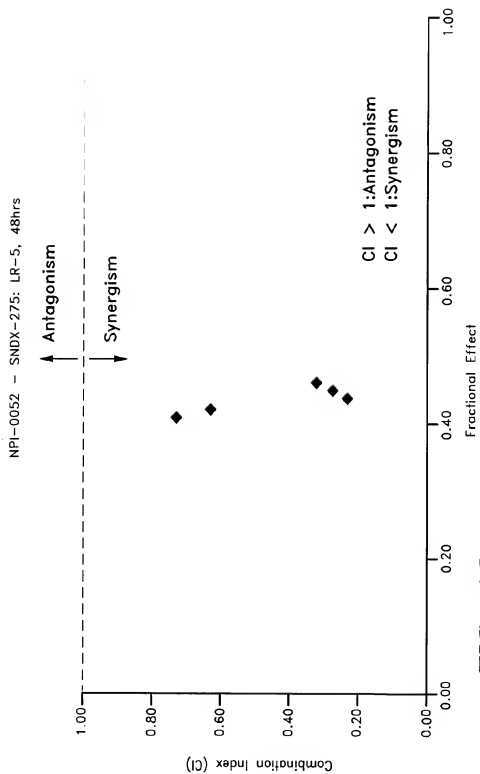


FIG. 16

34/61

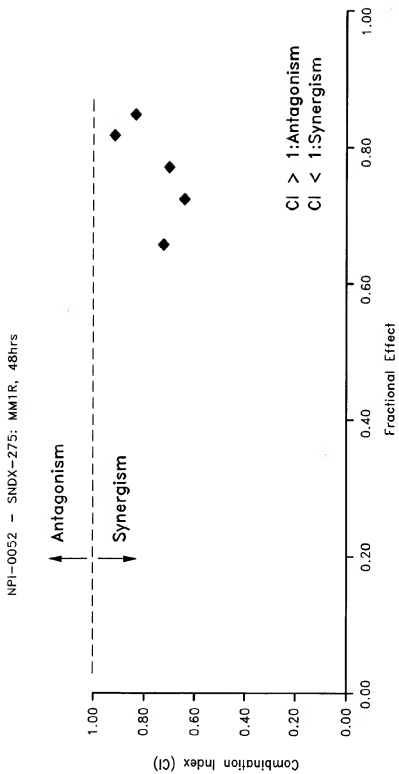
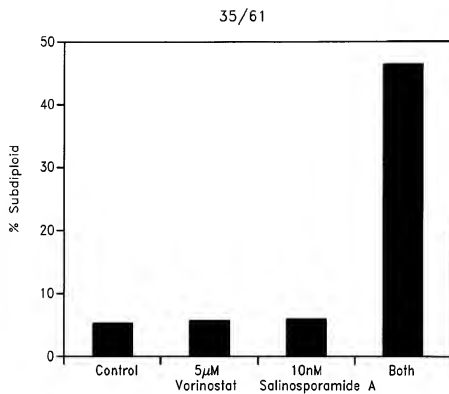
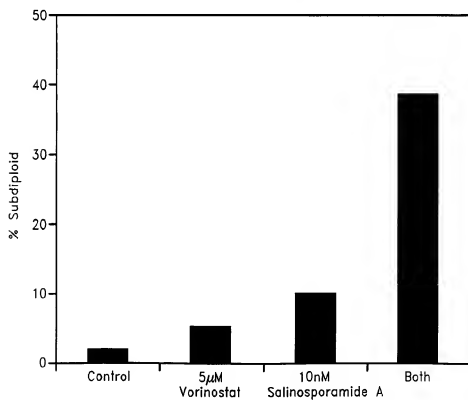
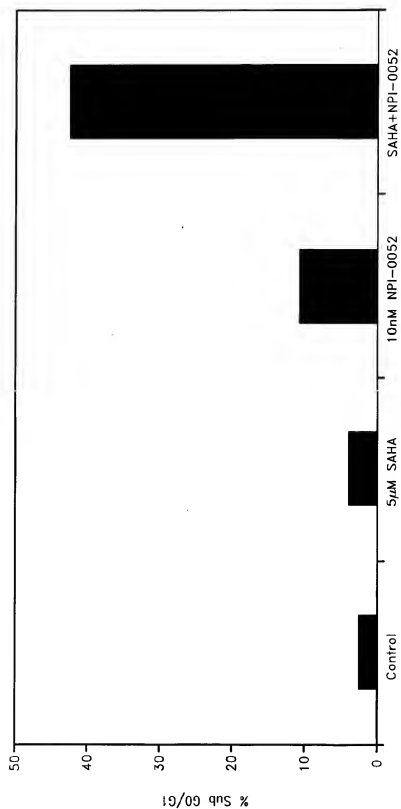


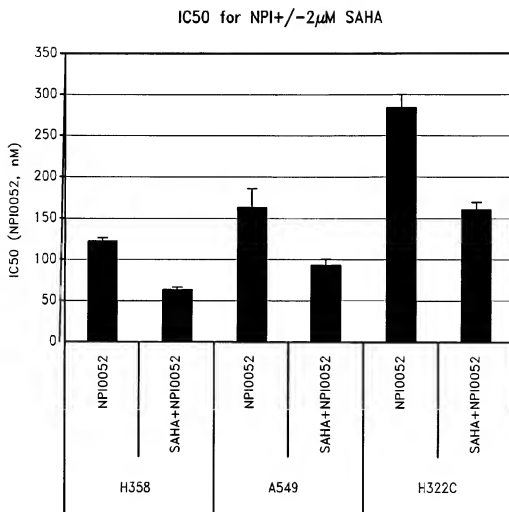
FIG. 17

*FIG. 18A**FIG. 18B*

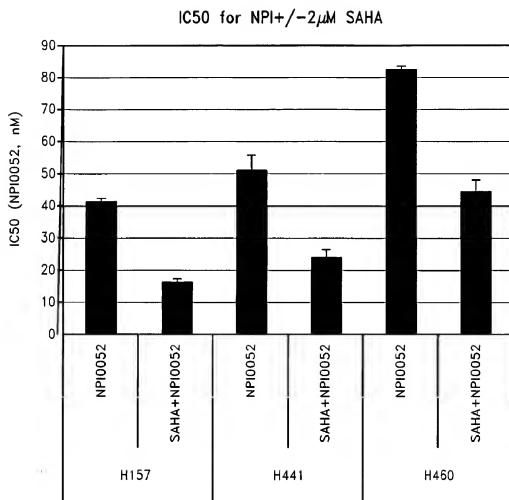
36/61

*FIG. 19*

37/61

*FIG. 20*

38/61

*FIG. 21*

39/61

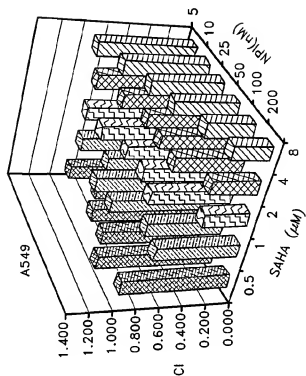


FIG. 22B

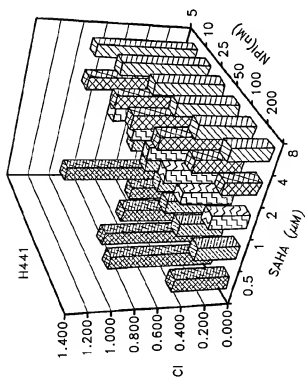


FIG. 22A

40/61

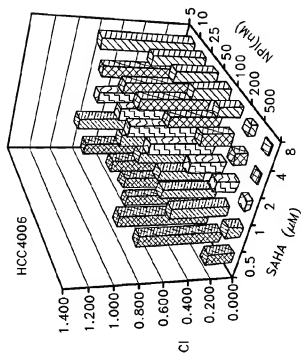


FIG. 22D

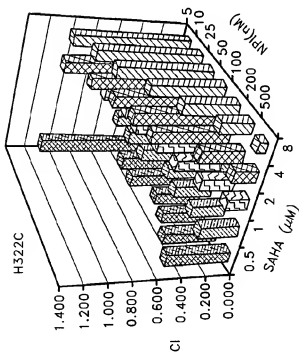


FIG. 22C

41/61

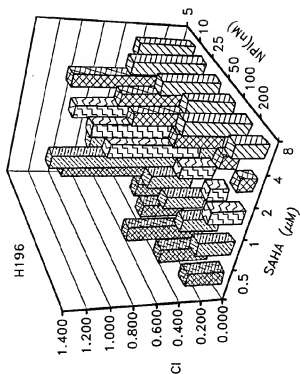


FIG. 22F

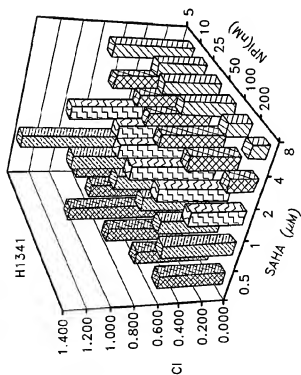


FIG. 22E

42/61

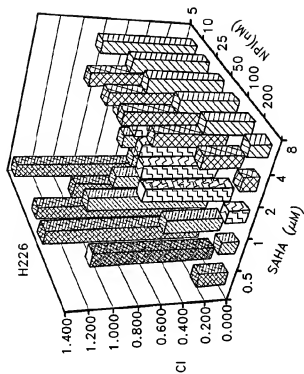


FIG. 22H

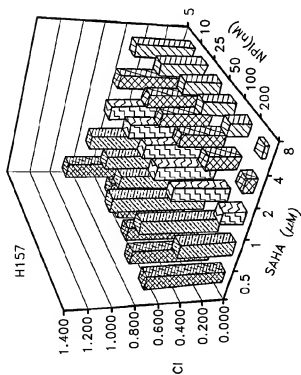


FIG. 22G

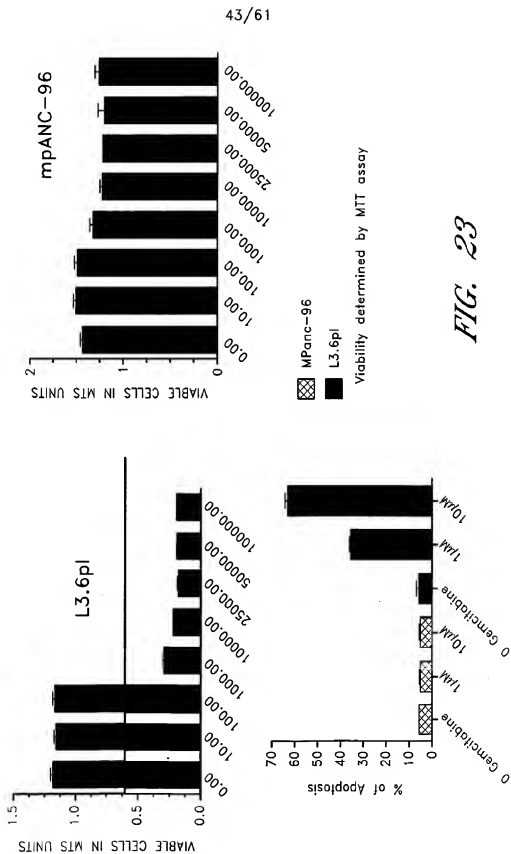
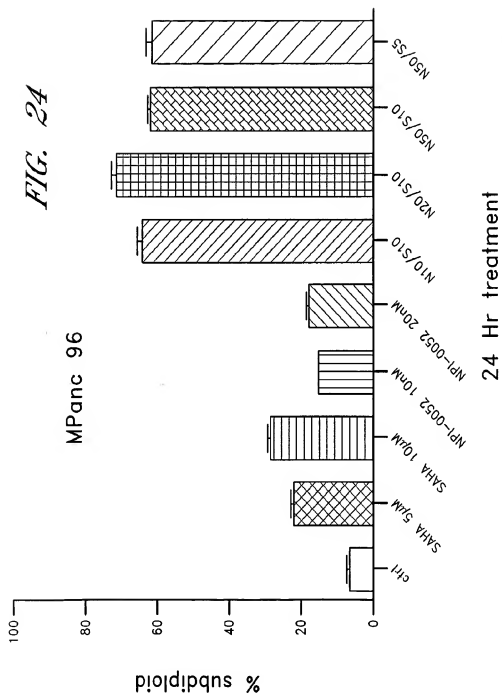


FIG. 23

44/61



45/61

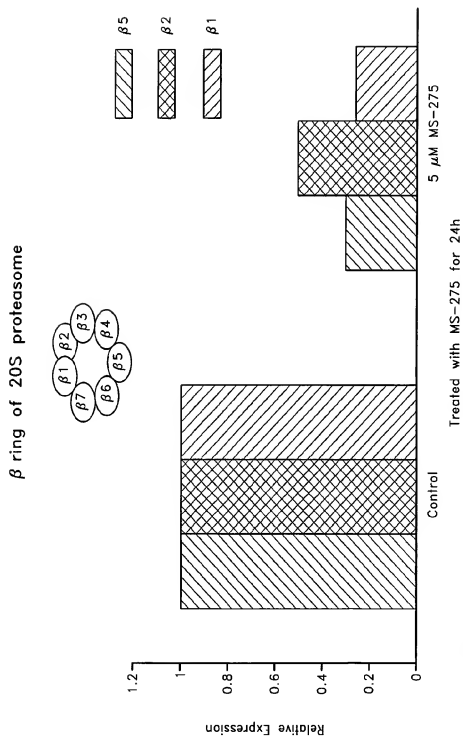
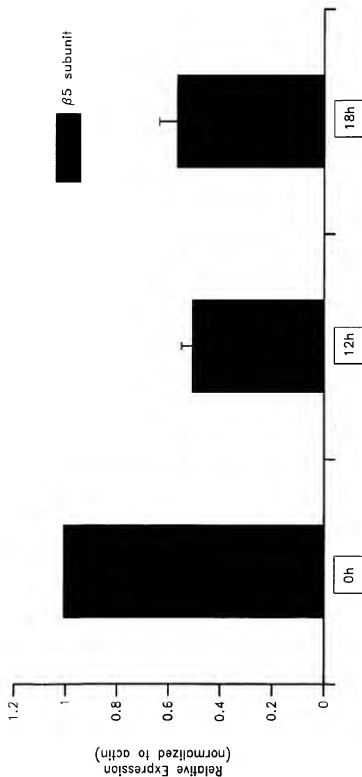


FIG. 25

46/61



- Time course of $\beta 5$ mRNA expression levels in Jurkat cells treated with 500 nM Vorinostat.
- Expression of $\beta 5$ mRNA was analyzed by real time PCR.

FIG. 26

47/61

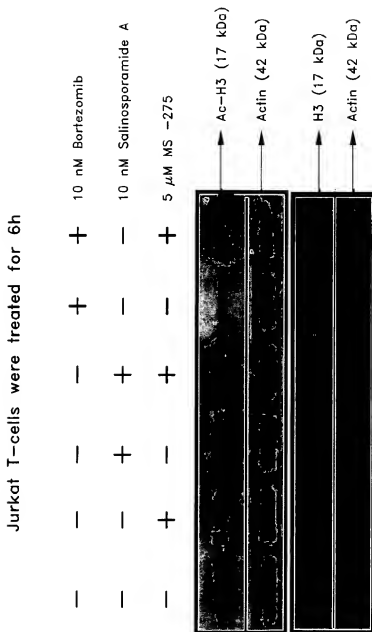
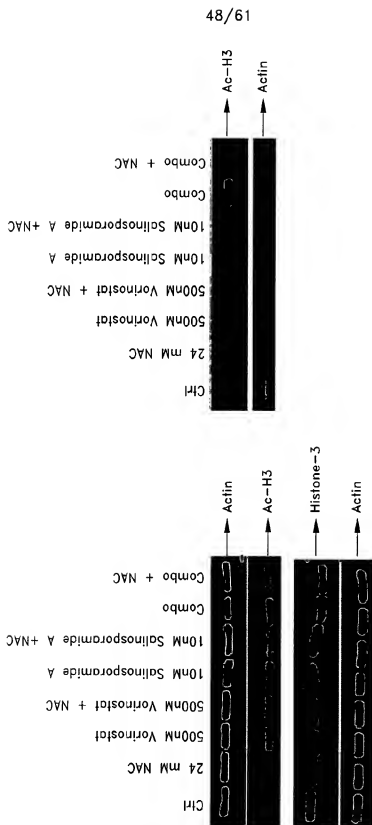


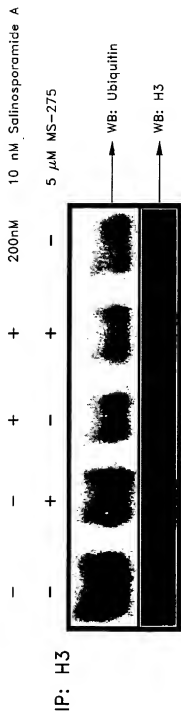
FIG. 27

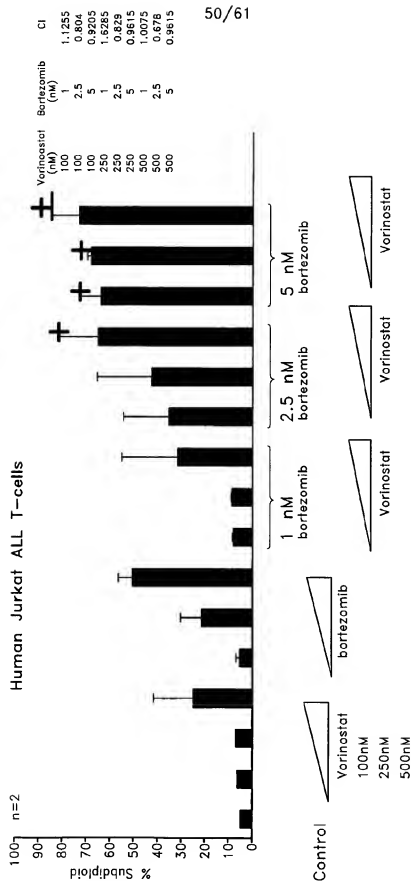


Cells were treated for 6h as indicated

FIG. 28

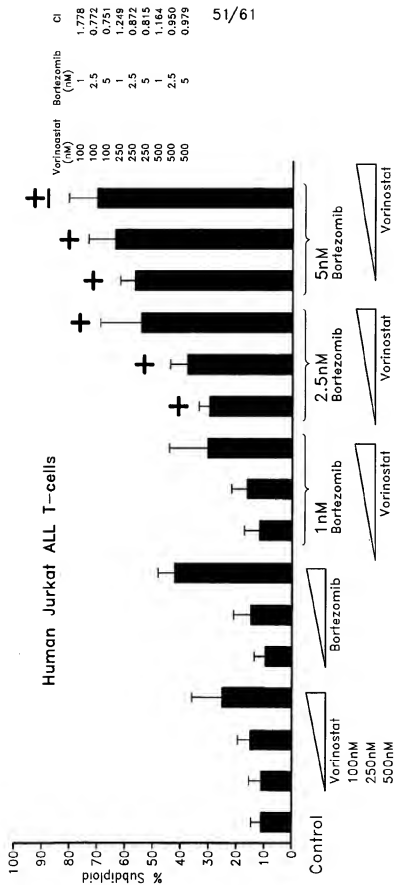
49/61

*FIG. 29*



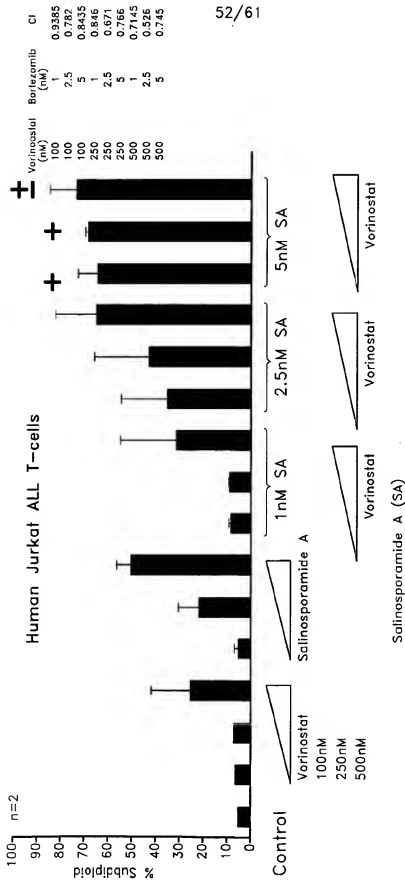
50/61

FIG. 30



5/61

FIG. 31



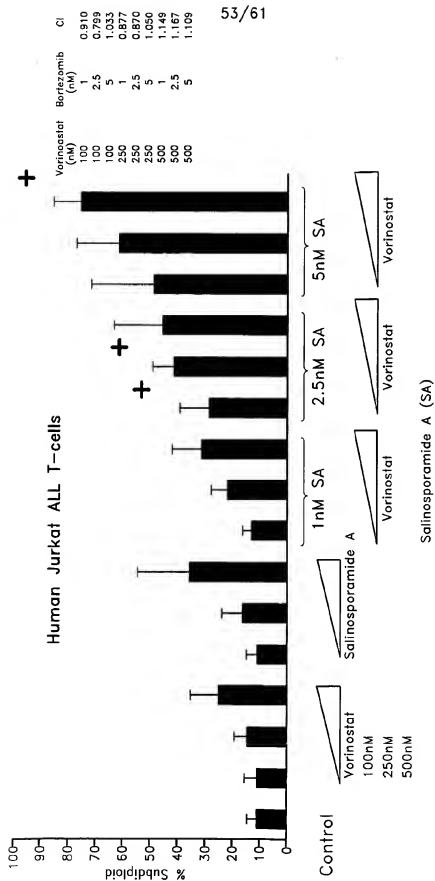


FIG. 33

54/61

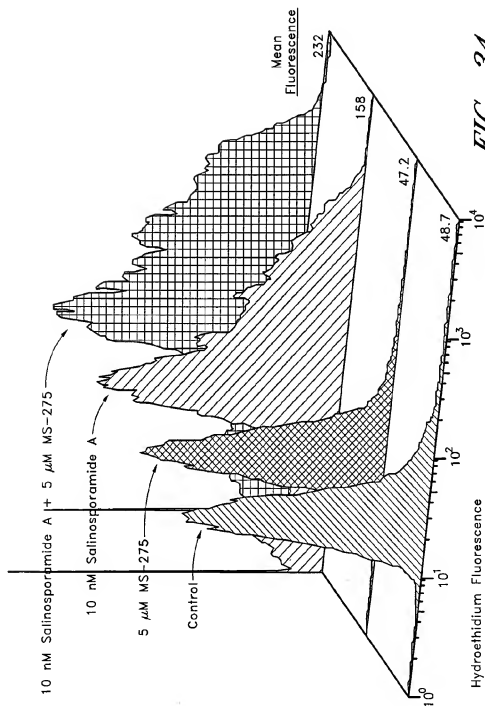
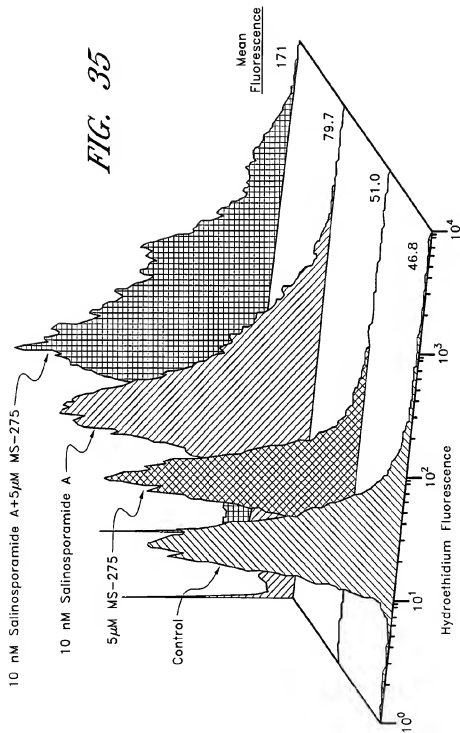


FIG. 3A

55/61

FIG. 35



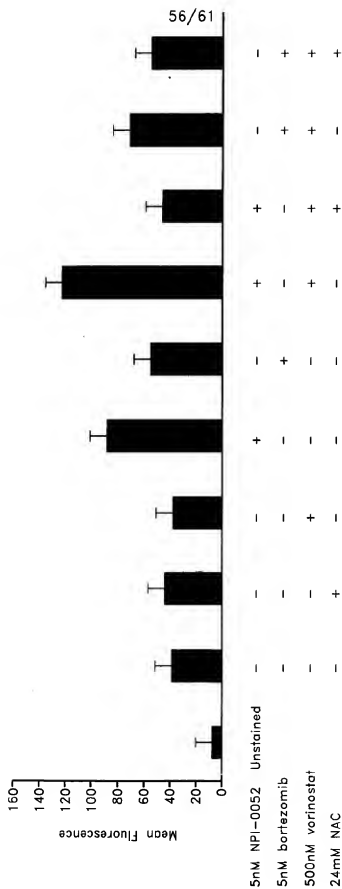
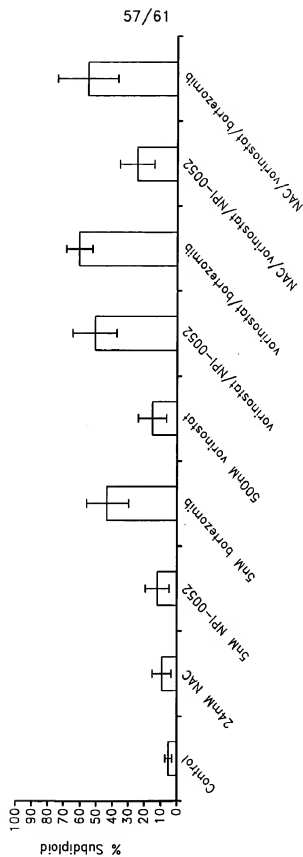
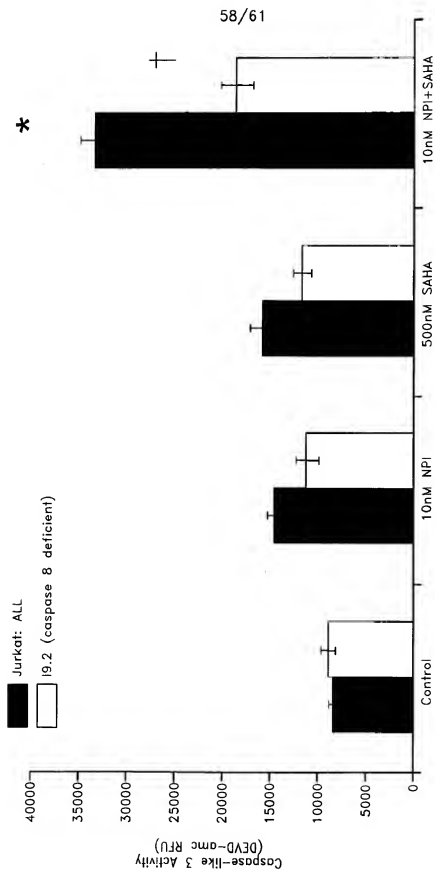


FIG. 36



NAC: N-acetylcysteine, a free radical scavenger

FIG. 37



* $P < 0.001$ (compared to Control on 10nM Salinosporamide A)
 † $P < 0.001$ (compared to Jurkat 10nM Salinosporamide A + Vorinostat)

FIG. 38

59/61

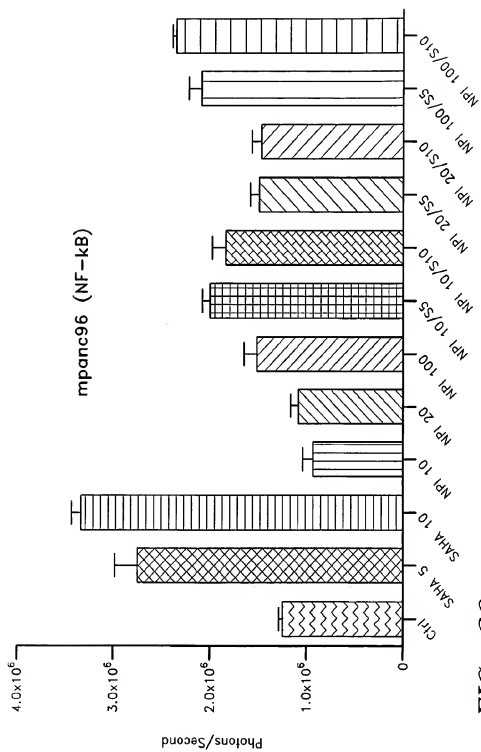
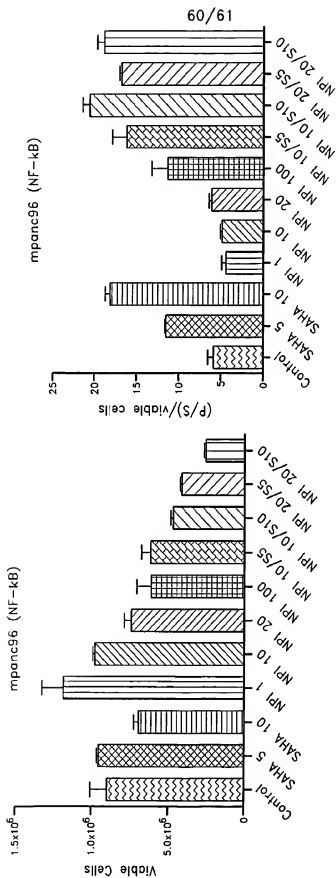


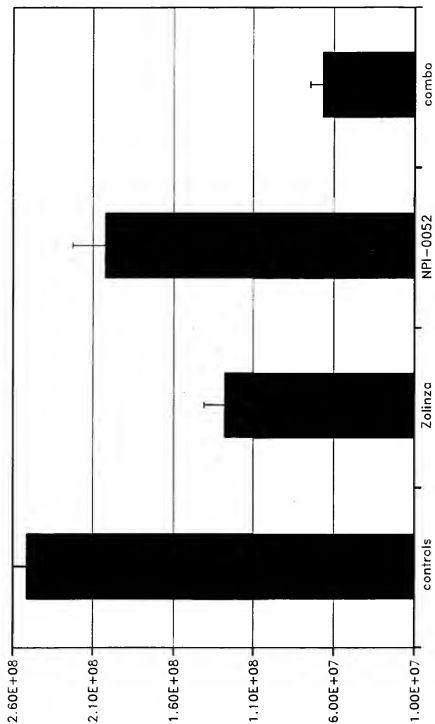
FIG. 39



Total NF-κB activity per well divided by the total viable cell count per well to standardize NF-κB activity per viable cell

FIG. 40

61/61

*FIG. 41*

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/059592

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/165	A61K31/166	A61K31/167	A61K31/19	A61K31/336
A61K31/40	A61K31/4045	A61K31/407	A61K31/427	A61K31/4406
A61K31/4439	A61K31/473	A61K45/06	A61P35/00	

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, WPI Data, BEILSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/060819 A (UNIV CALIFORNIA [US]; BONAVIDA BENJAMIN [US]) 8 June 2006 (2006-06-08) page 35, paragraphs 121,122; example 7	1-32
Y	claims 1,6,26	1-32
X	WO 2006/060676 A (DANA FARBER CANCER INST INC [US]; ANDERSON KENNETH C [US]; CHAUHAN DHA) 8 June 2006 (2006-06-08) claims 25-37; example 17	1-32
X	US 2005/288352 A1 (POTTS BARBARA C [US] ET AL POTTS BARBARA CHRISTINE [US] ET AL) 29 December 2005 (2005-12-29) paragraph [0331]; claims 1-39.	1-32

-/-

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

** document member of the same patent family

Date of the actual completion of the international search

27 August 2008

Date of mailing of the international search report

15/09/2008

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Renard, Delphine

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2008/059592

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/138196 A1 (FENICAL WILLIAM [US] ET AL FENICAL WILLIAM [US] ET AL) 15 July 2004 (2004-07-15) paragraph [0163]; claims 1-21	1-32
P,X	MITSIADIS ET AL: "From the bench to the bedside: emerging new treatments in multiple myeloma" BEST PRACTICE & RESEARCH CLINICAL HAEMATOLOGY, BAILLIÈRE TINDALL, vol. 20, no. 4, 21 December 2007 (2007-12-21), pages 797-816, XP022383314 ISSN: 1521-6926 page 801, lines 6,7 page 800, last paragraph page 803, last paragraph - page 804, last paragraph; compounds SAHA, LBH-589, TUBACIN	1-4, 10-16, 21,22, 27,28
Y	----- page 801, lines 6,7 page 800, last paragraph page 803, last paragraph - page 804, last paragraph; compounds SAHA, LBH-589, TUBACIN	1-32
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